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(54) Title: AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

#### (57) Abstract

A human gene has been discovered which is genetically altered in human tumor cells. The genetic alteration is gene amplification and leads to a corresponding increase in gene products. Detecting that the gene, designated hMDM2, has become amplified or detecting increased expression of gene products is diagnostic of tumorigenesis. Human MDM2 protein binds to human p53 and allows the cell to escape from p53-regulated growth.

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# AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

This application is a continuation-in-part of United States Serial No. 07/903,103, filed June 23, 1992, which is a continuation-in-part of United States Serial No. 07/867,840, filed April 7, 1992, now abandoned.

This invention was made with support from the U.S. Government, including NIH grants CA-57345, CA-43460, CA-02243 and CA-35494. Accordingly, the Government retains certain rights in the invention.

# FIELD OF THE INVENTION

The invention relates to the area of cancer diagnostics and therapeutics. More particularly, the invention relates to the detection of a gene which is amplified in certain human tumors.

# BACKGROUND OF THE INVENTION

According to the Knudson model for tumorigenesis (Cancer Research, 1985, vol. 45, p. 1482), there are tumor suppressor genes in all normal cells which, when they become non-functional due to mutation, cause neoplastic development. Evidence for this model has been found in cases of retinoblastoma and colorectal tumors. The implicated suppressor genes in these tumors, RB and p53 respectively, were found to be deleted or altered in many of the tumors studied.

The p53 gene product, therefore, appears to be a member of a group of proteins which regulate normal cellular proliferation and suppression of cellular transformation. Mutations in the p53 gene have been linked to tumorigenesis, suggesting that alterations

in p53 protein function are involved in cellular transformation. The inactivation of the p53 gene has been implicated in the genesis or progression of a wide variety of carcinomas (Nigro et al., 1989, Nature 342:705-708), including human colorectal carcinoma (Baker et al., 1989, Science 244:217-221), human lung cancer (Takahashi et al., 1989, Science 246:491-494; Iggo et al., 1990, Lancet 335:675-679), chronic myelogenous leukemia (Kelman et al, 1989, Proc. Natl. Acad. Sci. USA 86:6783-6787) and osteogenic sarcomas (Masuda et al., 1987, Proc. Natl. Acad. Sci. USA 84:7716-7719).

While there exists an enormous body of evidence linking p53 gene mutations to human tumorigenesis (Hollstein et al., 1991, *Science 253*:49-53) little is known about cellular regulators and mediators of p53 function.

Hinds et al. (*Cell Growth & Differentiation*, 1:571-580, 1990), found that p53 cDNA clones, containing a point mutation at amino acid residue 143, 175, 273 or 281, cooperated with the activated *ras* oncogene to transform primary rat embryo fibroblasts in culture. These mutant p53 genes are representative of the majority of mutations found in human cancer. Hollstein et al., 1991, *Science 253*:49-53. The transformed fibroblasts were found to produce elevated levels of human p53 protein having extended half-lives (1.5 to 7 hours) as compared to the normal (wild-type) p53 protein (20 to 30 minutes).

Mutant p53 proteins with mutations at residue 143 or 175 form an oligomeric protein complex with the cellular heat shock protein hsc70. While residue 273 or 281 mutants do not detectably bind hsc70, and are poorer at producing transformed foci than the 175 mutant, complex formation between mutant p53 and hsc70 is not required for p53-mediated transformation. Complex formation does, however, appear to facilitate this function. All cell lines transformed with the mutant p53 genes are tumorigenic in a thymic (nude) mice. In contrast, the wild-type human p53 gene does not possess transforming activity in cooperation with ras. Tuck and Crawford, 1989, Oncogene Res. 4:81-96.

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Hinds et al., supra also expressed human p53 protein in transformed rat cells. When the expressed human p53 was immunoprecipitated with two p53 specific antibodies directed against distinct epitopes of p53, an unidentified M<sub>r</sub> 90,000 protein was coimmunoprecipitated. This suggested that the rat M<sub>r</sub> 90,000 protein is in a complex with the human p53 protein in the transformed rat cell line.

As mentioned above, levels of p53 protein are often higher in transformed cells than normal cells. This is due to mutations which increase its metabolic stability (Oven et al., 1981, Mol. Cell. Biol. 1:101-110; Reich et al. (1983), Mol. Cell. Biol. 3:2143-2150). The stabilization of p53 has been associated with complex formation between p53 and viral or cellular proteins. (Linzer and Levine, 1979, Cell 17:43-52; Crawford et al., 1981, Proc. Natl. Acad. Sci. USA 78:41-45; Dippold et al., 1981, Proc. Natl. Acad. Sci. USA 78:1695-1699; Lane and Crawford, 1979, Nature (Lond.) 278:261-263; Hinds et al., 1987. Mol. Cell. Biol. 7:2863-2869; Finlay et al., 1988, Mol. Cell. Biol. 8:531-539; Sarnow et al., 1982, Cell. 28:387-394; Gronostajski et al., 1984, Mol. Cell. Biol. 4:442-448; Pinhasi-Kimhi et al., 1986, Nature (Lond.) 320:182-185; Ruscetti and Scolnick, 1983, J. Virol. 46:1022-1026; Pinhasi and Oren, 1984, Mol. Cell. Biol. 4:2180-2186; and Sturzbecher et al., 1987, Oncogene 1:201-211.) For example, p53 protein has been observed to form oligomeric protein complexes with the SV40 large T antigen, the adenovirus type 5 E1B-M, 55,000 protein, and the human papilloma virus type 16 or 18 E6 product. Linzer and Levine, 1979, Cell 17:43-52; Lane and Crawford, 1979, Nature, 278:261-263; Sarnow et al., 1982, Cell 28:387-394; Werness et al., 1990, Science, 248:76-79. Similarly, complexes have been observed of p105RB (the product of the retinoblastoma susceptibility gene) with T antigen (DeCaprio et al., 1988, Cell 54:275-283), the adenovirus EIA protein (Whyte et al., 1988, Nature 334:124-129) and the E7 protein of human papilloma virus 16 or 18 (Münger et al., 1989, EMBO J. 8:4099-4105). It has been suggested that interactions between these viral proteins and p105<sup>RB</sup> inactivate a growth-suppressive function of p105RB, mimicking deletions and mutations commonly found in the RB gene in tumor cells. In a similar fashion, oligomeric protein complex

formation between these viral proteins and p53 may eliminate or alter the function of p53. Finlay et al., 1989, Cell 57:1083-1093.

Fakharzadeh et al. (*EMBO J. 10*:1565-1569, 1991) analyzed amplified DNA sequences present in a tumorigenic mouse cell line (*i.e.*, 3T3DM, a spontaneously transformed derivative of mouse Balb/c cells). Studies were conducted to determine whether any of the amplified genes induced tumorigenicity following introduction of the amplified genes into a nontransformed recipient cell (*e.g.*, mouse NIH3T3 or Rat2 cells). The resulting cell lines were tested for tumorigenicity in nude mice. A gene, designated MDM2, which is amplified more than 50-fold in 3T3DM cells, induced tumorigenicity when overexpressed in NIH3T3 and Rat 2 cells. From the nucleotide and predicted amino acid sequence of mouse MDM2 (mMDM2), Fakharzadeh speculated that this gene encodes a potential DNA binding protein that functions in the modulation of expression of other genes and, when present in excess, interferes with normal constraints on cell growth.

#### SUMMARY OF THE INVENTION

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It is an object of the invention to provide a method for diagnosing a neoplastic tissue, such as sarcoma, in a human.

It is another object of the invention to provide a cDNA molecule encoding the sequence of human MDM2.

Yet another object of the invention is to provide a preparation of human MDM2 protein which is substantially free of other human cellular proteins.

Still another object of the invention is to provide DNA probes capable of hybridizing with human MDM2 genes or mRNA molecules.

Another object of the invention is to provide antibodies immunoreactive with human MDM2 protein.

Still another object of the invention is to provide kits for detecting amplification or elevated expression of human MDM2.

Yet another object of the invention is to provide methods for identifying compounds which interfere with the binding of human MDM2 to human p53.

A further object of the invention is to provide a method of treating a neoplastic human cell.

Yet another object of the invention is to provide methods for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification.

Still another object of the invention is to provide polypeptides which interfere with the binding of human MDM2 to human p53.

A further object of the invention is to provide a method for growing host cells containing a p53 expression vector.

It has now been discovered that hMDM2, a heretofore unknown human gene, plays a role in human cancer. The hMDM2 gene has been cloned and the recombinant derived hMDM2 protein shown to bind to human p53 in vitro. hMDM2 has been found to be amplified in some neoplastic cells and the expression of hMDM2-encoded products has been found to be correspondingly elevated in tumors with amplification of this gene. The elevated levels of MDM2 appear to sequester p53 and allow the cell to escape from p53-regulated growth.

# BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-C shows the cDNA sequence of human MDM2. In this figure, human and mouse nucleotide and amino acid sequences are compared, the mouse sequence being shown only where it differs from the corresponding human sequence.

Figure 2 shows that hMDM2 binds to p53.

Figure 3 illustrates the amplification of the hMDM2 gene in sarcomas.

Figure 4A-C illustrates hMDM2 expression.

Figure 5 shows the inhibition of p53-mediated transactivation by MDM2. Yeast were stably transfected with expression plasmids encoding p53, lex-VP16, MDM2 or the appropriate vector-only controls, as indicated. p53-responsive (bars a-c) or lexA-responsive (bars d-f)  $\beta$ -galactosidase reporter plasmids were used to assess the response.

Inset: Western blot analysis demonstrating MDM2 (90 kD) and p53 (53 kD) expression in representative yeast strains. The strain indicated by a plus was transfected with expression vector encoding full length MDM2 and p53, while the strain indicated by a minus was transfected only with the p53 expression vector.

Figure 6 shows the determination of MDM2 and p53 domains of interaction. Fig. 5A and Fig. 5B. Random fragments of MDM2 were fused to sequences encoding the lexA DNA binding domain and the resultant clones transfected into yeast carrying pRS314SN (p53 expression vector) and pJK103 (lexA-responsive  $\beta$ -galactosidase reporter). Yeast clones expressing  $\beta$ -galactosidase were identified by their blue color, and the MDM2 sequences in the lexA fusion vector were determined.  $\beta$ -galactosidase activity was observed independent of p53 expression in A, but was dependent on p53 expression in B. The bottom 6 clones in B were generated by genetic engineering. Fig. 6C. Random fragments of p53 were fused to the sequence encoding the B42 acidic activation domain and a hemagglutinin epitope tag; the resultant clones were transfected into yeast carrying lexA-MDM2 (lexA DNA binding domain fused to full length MDM2) and pJK103. Yeast clones were identified as above, and all were found to be MDM2-dependent. The bottom three clones were generated by genetic engineering.

Figure 7 shows protein expression from the yeast strains described in Figure 6. Western blot analysis was performed as described (Oliner, J.D., et al., *Nature 358*:80-83 (1992)), using 20 μg of protein per lane. The MDM2 and p53 codons contained in the fusion vectors are shown at the top of A and B, respectively. Fig. 7A. Upper panel probed with p53 Ab2 detecting p53; lower panel probed with anti-lexA polyclonal antibodies (lex Ab) detecting MDM2 fusion proteins of 30-50 kD. Fig. 7B. Upper panel probed with Lex Ab detecting the lexA-full length MDM2 fusion protein of 112 kD; lower panel probed with HA Ab (a monoclonal antibody directed against the hemagglutinin epitope tag, Berkeley Antibody) detecting p53 fusion proteins of approximately 25-30 kD.

Figure 8 shows the inhibition of the p53 activation domain by MDM2. Yeast were transfected with expression vectors encoding a lexA-p53 (p53 codons 1-73) fusion (bars a and b) or lexA alone (bar c). Strain b also expressed full length MDM2, and all strains contained the lexA-responsive  $\beta$ -galactosidase reporter plasmid. Inset: Upper panel probed with MDM2 polyclonal antibodies detecting full length MDM2 (90 kD); lower panel probed with lex Ab detecting the lex-p53 fusion protein of 40 kD.

Figure 9 shows a Western blot analysis using monoclonal antibodies to MDM2 or p53. Fifty  $\mu g$  of total cellular proteins from OsA-CL or SW480 cells were used for Western blot analysis. The position of molecular weight markers, in kd, is given on the right.

Figure 10 demonstrates immunocytochemical analysis of OsA-CL and SW480 cells grown *in vitro*. Monoclonal antibody IF-2, specific for MDM2, and mAb 1801, specific for p53, were used. The exclusively nuclear localization of both proteins is evident, as is the higher expression of MDM2 protein in OsA-CL cells than in SW480 cells, the reverse of the pattern observed for p53.

Figure 11 demonstrates MDM2 expression in primary soft tissue sarcomas. Cryostat sections of human sarcomas were incubated with the IF-2 antibody specific for MDM2. Tumors #3 and #10 showed nuclear expression of MDM2, while tumor #2 showed no staining.

# DETAILED DESCRIPTION OF THE INVENTION

It is a discovery of the present invention that a gene exists which is amplified in some human tumors. The amplification of this gene, designated MDM2, is diagnostic of neoplasia or the potential therefor. Detecting the elevated expression of human MDM2-encoded products is also diagnostic of neoplasia or the potential for neoplastic transformation. Over a third of the sarcomas surveyed, including the most common bone and soft tissue forms, were found to have amplified hMDM2 sequences. Expression of hMDM2 was found to be correspondingly elevated in tumors with the gene amplification.

Other genetic alterations leading to elevated hMDM2 expression may be involved in tumorigenesis also, such as mutations in regulatory regions of the gene. Elevated expression of hMDM2 may also be involved in tumors other than sarcomas including but not limited to those in which p53 inactivation has been implicated. These include colorectal carcinoma, lung cancer and chronic myelogenous leukemia.

According to one embodiment of the invention, a method of diagnosing a neoplastic tissue in a human is provided. Tissue or body fluid is isolated from a human, and the copy number of human MDM2 genes is determined. Alternatively, expression levels of human MDM2 gene products can be determined. These include protein and mRNA.

Body fluids which may be tested include urine, serum, blood, feces, saliva, and the like. Tissues suspected of being neoplastic are desirably separated from normal appearing tissue for analysis. This can be done by paraffin or cryostat sectioning or flow cytometry, as is known in the art. Failure to separate neoplastic from non-neoplastic cells can confound the analysis. Adjacent non-neoplastic tissue or any normal tissue can be used to determine a base-line level of expression or copy number, against which the amount of hMDM2 gene or gene products can be compared.

The human MDM2 gene is considered to be amplified if the cell contains more than the normal copy number (2) of this gene per genome. The various techniques for detecting gene amplification are well known in the art. Gene amplification can be determined, for example, by Southern blot analysis, as described in Example 4, wherein cellular DNA from a human tissue is digested, separated, and transferred to a filter where it is hybridized with a probe containing complementary nucleic acids. Alternatively, quantitative polymerase chain reaction (PCR) employing primers can be used to determine gene amplification. Appropriate primers will bind to sequences that bracket human MDM2 coding sequences. Other techniques for determining gene copy number as are known in the art can be used without limitation.

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The gene product which is measured may be either mRNA or protein. The term elevated expression means an increase in mRNA production or protein production over that which is normally produced by non-cancerous cells. Although amplification has been observed in human sarcomas, other genetic alterations leading to elevated expression of MDM2 may be present in these or other tumors. Other tumors include those of lung, breast, brain, colorectal, bladder, prostate, liver, skin, and stomach. These, too, are contemplated by the present invention. Non-cancerous cells for use in determining baseline expression levels can be obtained from cells surrounding a tumor, from other humans or from human cell lines. Any increase can have diagnostic value, but generally the mRNA or protein expression will be elevated at least about 3-fold, 5-fold, and in some cases up to about 100-fold over that found in non-cancerous cells. The particular technique employed for detecting mRNA or protein is not critical to the practice of the invention. Increased production of mRNA or protein may be detected, for example, using the techniques of Northern blot analysis or Western blot analysis, respectively, as described in Example 4 or other known techniques such as ELISA, immunoprecipitation, RIA and the like. These techniques are also well known to the skilled artisan.

According to another embodiment of the invention, nucleic acid probes or primers for the determining of human MDM2 gene amplification or elevated expression of mRNA are provided. The probe may comprise ribo- or deoxyribonucleic acids and may contain the entire human MDM2 coding sequence, a sequence complementary thereto, or fragments thereof. A probe may contain, for example, nucleotides 1-949, or 1-2372 as shown in Figure 1. Generally, probes or primers will contain at least about 14 contiguous nucleotides of the human sequence but may desirably contain about 40, 50 or 100 nucleotides. Probes are typically labelled with a fluorescent tag, a radioisotope, or the like to render them easily detectable. Preferably the probes will hybridize under stringent hybridization conditions. Under such conditions they will not hybridize to mouse MDM2. The probes of the invention are complementary to the human MDM2 gene. This means that they share 100% identity with the human sequence.

hMDM2 protein can be produced, according to the invention, substantially free of other human proteins. Provided with the DNA sequence, those of skill in the art can express the cDNA in a non-human cell. Lysates of such cells provide proteins substantially free of other human proteins. The lysates can be further purified, for example, by immunoprecipitation, co-precipitation with p53, or by affinity chromatography.

The antibodies of the invention are specifically reactive with hMDM2 protein. Preferably, they do not cross-react with MDM2 from other species. They can be polyclonal or monoclonal, and can be raised against native hMDM2 or a hMDM2 fusion protein or synthetic peptide. The antibodies are specifically immunoreactive with hMDM2 epitopes which are not present on other human proteins. Some antibodies are reactive with epitopes unique to human MDM2 and not present on the mouse homolog. The antibodies are useful in conventional analyses, such as Western blot analysis, ELISA, immunohistochemistry, and other immunological assays for the detection of proteins. Techniques for raising and purifying polyclonal antibodies are well known in the art, as are techniques for preparing monoclonal antibodies. Antibody binding can be determined by methods known in the art, such as use of an enzyme-labelled secondary antibody, staphylococcal protein A, and the like. Certain monoclonal antibodies of the invention have been deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852. These include IF2, and ED9, which have been granted accession nos. HB 11290, and HB 11291, respectively.

According to another embodiment of the invention, interference with the expression of MDM2 provides a therapeutic modality. The method can be applied in vivo, in vitro, or ex vivo. For example, expression may be down-regulated by administering triple-strand forming or antisense oligonucleotides which bind to the hMDM2 gene or mRNA, respectively, and prevent transcription or translation. The oligonucleotides may interact with unprocessed pre-mRNA or processed mRNA. Small molecules and peptides which specifically inhibit MDM2 expression can also be used.

Similarly, such molecules which inhibit the binding of MDM2 to p53 would be therapeutic by alleviating the sequestration of p53.

Such inhibitory molecules can be identified by screening for interference of the hMDM2/p53 interaction where one of the binding partners is bound to a solid support and the other partner is labeled. Antibodies specific for epitopes on hMDM2 or p53 which are involved in the binding interaction will interfere with such binding. Solid supports which may be used include any polymers which as known to bind proteins. The support may be in the form of a filter, column packing matrix, beads, and the like. Labeling of proteins can be accomplished according to any technique known in the art. Radiolabels, enzymatic labels, and fluorescent labels can be used advantageously. Alternatively, both hMDM2 and p53 may be in solution and bound molecules separated from unbound subsequently. Any separation technique known in the art may be employed, including immunoprecipitation or immunoaffinity separation with an antibody specific for the unlabeled binding partner.

It has been found that amino acid residues 13-41 of p53 (See SEQ ID NO:1) are necessary for the interaction of MDM-2 and p53. However, additional residues on either the amino or carboxy terminal side of the peptide appear also to be required. Nine to 13 additional p53 residues are sufficient to achieve MDM2 binding, although less may be necessary. Since cells which overexpress MDM2 escape from p53-regulated growth control in sarcomas, the use of p53-derived peptides to bind to excess MDM2 leads to reestablishment of p53-regulated growth control.

Suitable p53-derived peptides for administration are those which are circular, linear, or derivitized to achieve better penetration of membranes, for example. Other organic compounds which are modelled to achieve the same three dimensional structure as the peptide of the invention can also be used.

DNA encoding the MDM2-binding, p53-derived peptide, or multiple copies thereof, may also be administered to tumor cells as a mode of administering the peptide. The DNA will typically be in an expression construct, such as a retrovirus, DNA virus,

or plasmid vector, which has the DNA elements necessary for expression properly positioned to achieve expression of the MDM2-binding peptide. The DNA can be administered, *inter alia* encapsulated in liposomes, or in any other form known to the art to achieve efficient uptake by cells. As in the direct administration of peptide, the goal is to alleviate the sequestration of p53 by MDM2.

A cDNA molecule containing the coding sequence of hMDM2 can be used to produce probes and primers. In addition, it can be expressed in cultured cells, such as *E. coli*, to yield preparations of hMDM2 protein substantially free of other human proteins. The proteins produced can be purified, for example, with immunoaffinity techniques using the antibodies described above.

Kits are provided which contain the necessary reagents for determining gene copy number, such as probes or primers specific for the hMDM2 gene, as well as written instructions. The instructions can provide calibration curves to compare with the determined values. Kits are also provided to determine elevated expression of mRNA (i.e., containing probes) or hMDM2 protein (i.e., containing antibodies). Instructions will allow the tester to determine whether the expression levels are elevated. Reaction vessels and auxiliary reagents such as chromogens, buffers, enzymes, etc. may also be included in the kits.

The human MDM2 gene has now been identified and cloned. Recombinant derived hMDM2 has been shown to bind to human p53. Moreover, it has been found that hMDM2 is amplified in some sarcomas. The amplification leads to a corresponding increase in MDM2 gene products. Such amplification is associated with the process of tumorigenesis. This discovery allows specific assays to be performed to assess the neoplastic or potential neoplastic status of a particular tissue.

The following examples are provided to exemplify various aspects of the invention and are not intended to limit the scope of the invention.

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#### **EXAMPLES**

#### Example 1

To obtain human cDNA clones, a cDNA library was screened with a murine MDM2 (mMDM2) cDNA probe. A cDNA library was prepared by using polyadenylated RNA isolated from the human colonic carcinoma cell line CaCo-2 as a template for the production of random hexamer primed double stranded cDNA. Gubler and Hoffmann, 1983, Gene 25:263-268. The cDNA was ligated to adaptors and then to the lambda YES phage vector, packaged, and plated as described by Elledge et al. (Proc. Natl. Acad. Sci. USA, 88:1731-1735, 1991). The library was screened initially with a P-labelled (Kinzler, K.W., et al., Nucl. Acids Res. 17:3645-3653 (1989), Feinberg and Vogelstein, 1983, Anal. Biochem. 132:6-13) mMDM2 cDNA probe (nucleotides 259 to 1508 (Fakharzadeh et al., 1991, EMBO J. 10:1565-1569)) and then rescreened with an hMDM2 cDNA clone containing nucleotides 40 to 702.

Twelve clones were obtained, and one of the clones was used to obtain thirteen additional clones by re-screening the same library. In total, twenty-five clones were obtained, partially or totally sequenced, and mapped. Sequence analysis of the twenty-five clones revealed several cDNA forms indicative of alternative splicing. The sequence shown in Figure 1 is representative of the most abundant class and was assembled from three clones: c14-2 (nucleotides 1-949), c89 (nucleotides 467-1737), and c33 (nucleotides 390-2372). The 3' end of the untranslated region has not yet been cloned in mouse or human. The 5' end is likely to be at or near nucleotide 1. There was an open reading frame extending from the 5' end of the human cDNA sequence to nucleotide 1784. Although the signal for translation initiation could not be unambiguously defined, the ATG at nucleotide 312 was considered the most likely position for several reasons. First, the sequence similarity between hMDM2 and mMDM2 fell off dramatically upstream of nucleotide 312. This lack of conservation in an otherwise highly conserved protein suggested that the sequences upstream of the divergence may not code for protein. Second, an anchored polymerase chain reaction (PCR) approach was employed in an

effort to acquire additional upstream cDNA sequence. Ochman et al., 1985, In: PCR Technology: Principles and Applications for DNA Amplification (Erlich, ed.) pp. 105-111 (Stockton, New York). The 5' ends of the PCR derived clones were very similar (within 3 bp) to the 5' ends of clones obtained from the cDNA library, suggesting that the 5' end of the hMDM2 sequence shown in Figure 1 may represent the 5' end of the transcript. Third, in vitro translation of the sequence shown in Figure 1, beginning with the methionine encoded by the nucleotide 312 ATG, generated a protein similar in size to that observed in human cells.

In Figure 1, hMDM2 cDNA sequence, hMDM2 and mMDM2 nucleotide and amino acid sequences are compared. The mouse sequence is only shown where it differs from the corresponding human sequence. Asterisks mark the 5' and 3' boundaries of the previously published mMDM2 cDNA. Fakharzadeh et al., 1991, EMBO J. 10:1565-1569. Dashes indicate insertions. The mouse and human amino acid sequences are compared from the putative translation start site at nucleotide 312 through the conserved stop codon at nucleotide 1784.

Comparison of the human and mouse MDM2 coding regions revealed significant conservation at the nucleotide (80.3%) and amino acid (80.4%) levels. Although hMDM2 and mMDM2 bore little similarity to other genes recorded in current databases, the two proteins shared several motifs. These included a basic nuclear localization signal (Tanaka, 1990, FEBS Letters 271:41-46) at codons 181 to 185, several casein kinase II serine phosphorylation sites (Pinna, 1990, Biochem. et. Biophys. Acta. 1054:267-284) at codons 166 to 169, 192 to 195, 269 to 272, and 290 to 293, an acidic activation domain (Ptashne, 1988, Nature 355:683-689) at codons 223 to 274, and two metal binding sites (Harrison, 1991, Nature 353:715) at codons 305 to 322 and 461 to 478, neither of which is highly related to known DNA binding domains. The protein kinase A domain noted in mMDM2 (Fakharzadeh et al., 1991, EMBO J. 10:1565-1569) was not conserved in hMDM2.

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#### Example 2

To determine whether the hMDM2 protein could bind to human p53 protein in vitro, an hMDM2 expression vector was constructed from the cDNA clones. The hMDM2 expression vector was constructed in pBluescript SK+ (Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in Figure 1 from nucleotide 312 to 2176. A 42 bp black bettle virus ribosome entry sequence (Dasmahapatra et al., 1987, Nucleic Acid Research 15:3933) was placed immediately upstream of this hMDM2 sequence in order to obtain a high level of expression. This construct, as well as p53 (El-Deriy et al., 1992, Nature Genetics, in press) and MCC (Kinzler et al., 1991, Science 251:1366-1370) constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Although the predicted size of the protein generated from the construct was only 55.2 kd (extending from the methionine at nucleotide 312 to nucleotide 1784), in vitro translated protein migrated at approximately 95 kilodaltons.

Ten  $\mu$ l of lysate containing the three proteins (hMDM2, p53 and MCC), alone or mixed in pairs, were incubated at 37°C for 15 minutes. One microgram (10  $\mu$ l) of p53 Ab1 (monoclonal antibody specific for the C-terminus of p53) or Ab2 (monoclonal antibody specific for the N-terminus of p53) (Oncogene Science), or 5  $\mu$ l of rabbit serum containing MDM2 Ab (polyclonal rabbit anti-hMDM2 antibodies) or preimmune rabbit serum (obtained from the rabbit which produced the hMDM2 Ab), were added as indicated. The polyclonal rabbit antibodies were raised against an *E. coli*-produced hMDM2-glutathione S-transferase fusion protein containing nucleotides 390 to 816 of the hMDM2 cDNA. Ninety  $\mu$ l of RIPA buffer (10 mM tris [pH 7.5], 1% sodium deoxycholate, 1% NP40, 150 mM NaCl, 0.1% SDS), SNNTE buffer, or Binding Buffer (El-Deriy et al., 1992, *Nature Genetics*, in press) were then added and the mixtures allowed to incubate at 4°C for 2 hours.

Two milligrams of protein A sepharose were added to each tube, and the tubes were rotated end-over-end at 4°C for 1 hour. After pelleting and washing, the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and the dried gels autoradiographed for 10 to 60 minutes in the presence of Enhance (New England Nuclear).

Figure 2 shows the co-precipitation of hMDM2 and p53. The three buffers produced similar results, although the co-precipitation was less efficient in SNNTE buffer containing 0.5 M NaCl (Figure 2, lanes 5 and 8) than in Binding Buffer containing 0.1 M NaCl (Figure 2 lanes 6 and 9).

In vitro translated hMDM2, p53 and MCC proteins were mixed as indicated above and incubated with p53 Ab1, p53 Ab2, hMDM2 Ab, or preimmune serum. Lanes 1, 4, 7, 10 and 14 contain aliquots of the protein mixtures used for immunoprecipitation. The bands running slightly faster than p53 are polypeptides produced from internal translation initiation sites.

The hMDM2 protein was not immunoprecipitated with monoclonal antibodies to either the C-terminal or N-terminal regions of p53 (Figure 2, lanes 2 and 3). However, when *in vitro* translated human p53 was mixed with the hMDM2 translation product, the anti-p53 antibodies precipitated hMDM2 protein along with p53, demonstrating an association *in vitro* (Figure 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility from another gene (MCC (Kinzler et al., 1991, *Science* 251:1366-1370)) was mixed with p53. No co-precipitation of the MCC protein was observed (Figure 2, lanes 8 and 9). When an *in vitro* translated mutant form of p53 (175his) was mixed with hMDM2 protein, a similar co-precipitation of hMDM2 and p53 proteins was also observed.

In the converse of the experiments described above, the anti-hMDM2 antibodies immunoprecipitated p53 when mixed with hMDM2 protein (Figure 2, lane 15) but failed to precipitate p53 alone (Figure 5, lane 13). Preimmune rabbit serum failed to precipitate either hMDM2 or p53 (Figure 2, lane 16).

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#### Example 3

In order to ascertain the chromosomal localization of hMDM2, somatic cell hybrids were screened with an hMDM2 cDNA probe. A human-hamster hybrid containing only human chromosome 12 was found to hybridize to the probe. Screening of hybrids containing portions of chromosome 12 (Turc-Carel et al., 1986, Cancer Genet. Cytogenet. 23:291-299) with the same probe narrowed the localization to chromosome 12q12-14.

#### Example 4

Previous studies have shown that this region of chromosome 12 is often aberrant in human sarcomas. Mandahl et al., 1987, Genes Chromosomes & Cancer 1:9-14; Turc-Carel et al., 1986, Cancer Genet. Cytogenet. 23:291-299; Meltzer et al., 1991, Cell Growth & Differentiation 2:495-501. To evaluate the possibility that hMDM2 was genetically altered in such cancers, Southern blot analysis was performed.

Figure 3 shows examples of the amplification of the hMDM2 gene in sarcomas. Cellular DNA (5 μg) was digested with *Eco*RI, separated by agarose gel electrophoresis, and transferred to nylon as described by Reed and Mann (*Nucl. Acids Res., 1985, 13*:7207-7215). The cellular DNA was derived from five primary sarcomas (lanes 1-4, 6) and one sarcoma cell line (OsA-C1, lane 5). The filters were then hybridized with an hMDM2 cDNA fragment probe nucleotide 1-949 (see Figure 1), or to a control probe which identifies fragments of similar size (DCC gene, 1.65 cDNA fragment). Fearon, 1989, *Science 247*:49-56. Hybridization was performed as described by Vogelstein et al. (*Cancer Research, 1987, 47*:4806-4813). A striking amplification of hMDM2 sequences was observed in several of these tumors. (See Figure 3, lanes 2, 3 and 5). Of 47 sarcomas analyzed, 17 exhibited hMDM2 amplification ranging from 5 to 50 fold. These tumors included 7 to 13 liposarcomas, 7 of 22 malignant fibrous histiocytomas (MFH), 3 of 11 osteosarcomas, and 0 and 1 rhabdomyosarcomas. Five benign soft tissue tumors (lipomas) and twenty-seven carcinomas (colorectal or gastric) were also tested by Southern blot analysis and no amplification was observed.

#### Example 5

This example illustrates that gene amplification is associated with increased expression.

Figure 4A illustrates hMDM2 expression as demonstrated by Northern blot analysis. Because of RNA degradation in the primary sarcomas, only the cell lines could be productively analyzed by Northern blot. RNA was separated by electrophoresis in a MOPS-formaldehyde gel and electrophoretically transferred to nylon filters. Transfer and hybridization were performed as described by Kinzler et al. (*Nature 332*:371-374, 1988). The RNA was hybridized to the hMDM2 fragment described in Figure 3. Ten  $\mu$ g of total RNA derived, respectively, from two sarcoma cell lines (OsA-CL, lane 1 and RC13, lane 2) and the colorectal cancer cell line (CaCo-2) used to make the cDNA library (lane 3). Lane 4 contains 10  $\mu$ g of polyadenylated CaCo-2 RNA. RNA sizes are shown in kb. In the one available sarcoma cell line with hMDM2 amplification, a single transcript of approximately 5.5 kb was observed (Figure 4A, lane 1). The amount of this transcript was much higher than in a sarcoma cell line without amplification (Figure 4A, lane 2) or in a carcinoma cell line (Figure 4A, lane 3). When purified mRNA (rather than total RNA) from the carcinoma cell line was used for analysis, an hMDM2 transcript of 5.5 kb could also be observed (Figure 4A, lane 4).

Figure 4B illustrates hMDM2 expression as demonstrated by Western blot analysis of the sarcoma cell lines RC13 (lane 1), OsA-CL (lane 3), HOS (lane 4), and the carcinoma cell line CaCo-2 (lane 2).

Figure 4C illustrates hMDM2 expression as demonstrated by Western blot analysis of primary sarcomas. Lanes 1 to 3 contain protein from sarcomas with hMDM2 amplifications, and lanes 4 and 5 contain protein from sarcomas without hMDM2 amplification.

Western blots using affinity purified MDM2 Ab were performed with 50  $\mu$ g protein per lane as described by Kinzler et al. (Mol. Cell. Biol., 1990, 10:634-642), except that the membranes were blocked in 10% nonfat dried milk and 10% goat serum,

and secondary antibodies were coupled to horseradish peroxidase, permitting chemiluminescent detection (Amersham ECL). MDM2 Ab was affinity purified with a pATH-hMDM2 fusion protein using methods described in Kinzler et al. (*Mol. Cell. Biol. 10*:634-642, 1990). Non-specifically reactive proteins of about 75-85, 105-120 and 170-200 kd were observed in all lanes, irrespective of hMDM2 amplification status. hMDM2 proteins, of about 90-97 kd, were observed only in the hMDM2-amplified tumors. Protein marker sizes are shown in kd.

A protein of approximately 97 kilodaltons was expressed at high levels in the sarcoma cell line with hMDM2 amplification (Figure 4B, lane 3), whereas no expression was evident in two sarcoma cell lines without amplification or in the carcinoma cell line (Figure 4B, lanes 1, 2 and 4). Five primary sarcomas were also examined by Western blot analysis. Three primary sarcomas with amplification expressed the same size protein as that observed in the sarcoma cell line (Figure 4C, lanes 1-3), while no protein was observed in the two sarcomas without amplification (Figure 4C, lanes 4 and 5).

Expression of the hMDM2 RNA in the sarcoma with amplification was estimated to be at least 30 fold higher than that in the other lines examined. This was consistent with the results of Western blot analysis.

The above examples demonstrate that hMDM2 binds to p53 in vitro and is genetically altered (i.e., amplified) in a significant fraction of sarcomas, including MFH, liposarcomas, and osteosarcomas. These are the most common sarcomas of soft tissue and bone. Weiss and Enzinger, 1978, Cancer 41:2250-2266; Malawer et al., 1985, In: Cancer: Principles and Practice of Oncology, DeVita et al., Eds., pp. 1293-1342 (Lippincott, Philadelphia).

Human MDM2 amplification is useful for understanding the pathogenesis of these often lethal cancers.

MDM2 may functionally inactivate p53 in ways similar to those employed by virally encoded oncoproteins such as SV40 T-antigen, adenovirus E1B, and HPV E6. Lane and Bechimol, 1990, Genes and Development 4:1-8; Werness et al., 1990, Science

248:76. Consistent with this hypothesis, no sarcomas with hMDM2 amplification had any of the p53 gene mutations that occur commonly in other tumors. hMDM2 amplification provides a parallel between viral carcinogenesis and the naturally occurring genetic alterations underlying sporadic human cancer. The finding that expression of hMDM2 is correspondingly elevated in tumors with amplification of the gene are consistent with the finding that MDM2 binds to p53, and with the hypothesis that overexpression of MDM2 in sarcomas allows escape from p53 regulated growth control. This mechanism of tumorigenesis has striking parallels to that previously observed for virally induced tumors (Lane and Bechimol, 1990, Genes and Development 4:1-8; Werness et al., 1990, Science 248:76), in which viral oncogene products bind to and functionally inactivate p53.

#### Example 6

This example demonstrates that MDM2 expression inhibits p53-mediated transactivation.

To determine if MDM2 could influence the ability of p53 to activate transcription, expression vectors coding for the two proteins were stably transfected into yeast along with a p53-responsive reporter construct. The reporter consisted of a β-galactosidase gene under the transcriptional control of a minimal promoter and a multimerized human DNA sequence which strongly bound p53 in vitro (Kern, S.E., et al., Science 256:827-830 (1992). Reporter expression was completely dependent on p53 in this assay (Figure 5, compare bars a and c). MDM2 expression was found to inhibit p53-mediated transactivation of this reporter 16-fold relative to isogeneic yeast lacking MDM2 expression (Figure 5, compare bars a and b). Western blot analysis confirmed that p53 (53 kD) was expressed equivalently in strains with and without MDM2 (90 kD) (Figure 1, inset).

METHODS. The MDM2 expression plasmid, pPGK-MDM2, was constructed by inserting the full length MDM2 cDNA (Oliner, J.D., et al., Nature 358:80-83 (1992)) into pPGK (Poon, D. et al., Mol. and Cell.

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Biol. 1111:4809-4821 (1991)), immediately downstream of the phosphoglycerate kinase constitutive promoter. Galactose-inducible p53 (pRS314SN, Nigro, J.M., et al., Mol. and Cell. Biol. 12:1357-1365 (1992)), lexA-VP16 (YVLexA, Dalton, S., et al., Cell 68:597-612 (1992)), and lexA (YLexA, YVLexA minus VP16) plasmids were used as indicated. The reporters were PG16-lacZ (Kern, S.E. et al., Science 256:827-830 (1992)) (p53-responsive) and pJK103 (Kamens, J., et al., Mol. Cell. Biol. 10:2840-2847 (1990)) (lexA-responsive). S. cerevisiae strain pEGY48 was transformed as described (Kinzler, K.W. et al., Nucl. Acids Res. 17:3645-3653 (1989)). Yeast strains represented by bars a-c were grown at 30°C to mid-log phase in selective liquid medium containing 2% raffinose as the carbon source, induced for 30 minutes by the addition of 2% galactose, harvested, and lysed as described (Kern, S.E. et al., Science 256:827-830 (1992)). The strains represented by bars d-f were treated similarly, except that the cells were induced in galactose for 4 hours to obtain measurable levels of  $\beta$ -galactosidase.  $\beta$ -galactosidase activities shown represent the mean of three to five experimental values (error bars indicate s.e.m.). Protein concentrations were determined by a Coomassie blue-based (bio-Rad) assay. The  $\beta$ -galactosidase assays were performed with AMPGD chemiluminescent substrate and Emerald enhancer (Tropix) according to the manufacturer's instructions. galactosidase activities of bars b and c are shown relative to that of bar A;  $\beta$ -galactosidase activities of bars e and f are shown relative to that of bar d. Western blots were performed as described (Oliner, J.D., et al., Nature 358:80-83 (1992)), using p53 Ab1801 (lower panel, Oncogene Science) or MDM2 polyclonal antibodies (Oliner, J.D., et al., Nature 358:80-83 (1992)) (upper panel).

To ensure that this inhibition was not simply a general transcriptional down regulation mediated by the expression of the foreign MDM2 gene, a yeast strain was created that contained a different transcriptional activator (lexA-VP16, consisting of the lexA DNA binding domain fused to the VP16 acidic activation domain), a similar reporter (with a lexA-responsive site upstream of a  $\beta$ -galactosidase gene), and the same MDM2 expression vector. The results shown in Figure 1 (bars d & e) demonstrate that lexA-VP16 transactivation was unaffected by the presence of MDM2. Furthermore, MDM2 expression had no apparent effect on the growth rate of the cells.

#### Example 7

This example demonstrates the domains of p53 and MDM2 which interact with each other.

To gain insight into the mechanism of the MDM2-mediated p53 inhibition, the domains of MDM2 and p53 responsible for binding to one another were mapped. The yeast system used to detect protein-protein binding takes advantage of the modular nature of transcription factor domains (Keegan, L., et al., Science 231:699-704 (1986); Chien, C.-T., Proc. Natl. Acad. Sci. U.S.A. 88:9578-9582 (1991); Brent, R., et al., Cell 43:729-731 (1985); Ma, J., et al., Cell 55:4430446 (1988). Generically, if protein 1 (fused to a sequence-specific DNA binding domain) is capable of binding to protein 2 (fused to a transcriptional activation domain), then co-expression of both fusion proteins will result in transcriptional activation of a suitable reporter. In our experiments, the lexA DNA binding domain (amino acids 2-202) and the B42 acidic activation domain (AAD) were used in the fusion constructs. The reporter (Kamens, J., et al., Mol. Cell. Biol. 10:2840-2847 (1990); contained a lexA-responsive site upstream of a  $\beta$ galactosidase gene. As an initial control experiment, full length MDM2 was inserted into the lexA fusion vector, and full length p53, supplying its intrinsic activation domain was inserted into a non-fusion vector. The combination resulted in the activation of the lexAresponsive reporter, while the same expression constructs lacking either the MDM2 or p53 cDNA inserts failed to activate  $\beta$ -galactosidase (Table I, strains 1, 2, and 3). Thus, activation was dependent upon MDM2-p53 binding.

This assay was then applied to mapping the interaction domains of each protein. Full length cDNA fragments encoding MDM2 or p53 were randomly sheared by sonication, amplified by polymerase chain reaction, size fractionated, cloned into the appropriate fusion vectors and transfected into yeast along with the reporter and the full length version of the other protein.

METHODS. Full length MDM2 cDNA in pBluescript SK+(Stratagene) was digested with XhoI and BamHI to excise the entire insert. agarose gel purification, the insert was sheared into random fragments by sonication, polished with the Klenow fragment of DNA polymerase I, ligated to catch linkers, and amplified by the polymerase chain reaction as described (Kinzler, K.W., et al., Nucl. Acids Res. 17:3645-3653 (1989)). The fragments were fractionated on an acrylamide gel into size ranges of 100-400 bp or 400-1000 pb, cloned into lexA(1-202)+PL (Ruden, D.M., et al., Nature 350:250-252 (1991)), and transfected into bacteria (XL-1 Blue, Stratagene). At least 10,000 bacterial colonies were scraped off agar plates, and the plasmid DNA was transfected into a strain of pEGY48 containing pRS314N (p53 expression vector) and pJK103 (lexA-responsive  $\beta$ -galactosidase reporter). Approximately 5,000 yeast clones were plated on selective medium containing 2% dextrose, and were replica-plated onto glalctose- and X-gal-containing selective medium. Blue colonies (17) appeared only on the plates containing the larger fragments of MDM2. The 17 isolated colonies were tested for blue color in this assay both in the presence and in the absence of galactose (p53 induction); all tested positive in the presence of galactose but only 2 of the 17 tested positive in its absence. MDM2-containing plasmid DNA extracted from the 17 yeast clones was selectively transferred to bacterial strain KC8 and sequenced from the lexA-MDM2 junction. The MDM2 sequences of the two p53independent clones are diagrammed in Fig. 6A. The MDM2 sequences of the remaining 15 p53-dependent clones coded for peptides ranging from 135 to 265 a.a. in length and began exclusively at the initiator methionine. Three of the MDM2 sequences obtained are shown at the top of Fig. 6B. The lower 6 sequences were genetically engineered (using the polymerase chain reaction and appropriate primers) into lexA(1-202)+PL and subsequently tested to further narrow the binding region.

Fragments of p53 were also cloned into pJG4-5, producing a fusion protein C-terminal to the B42 acidic activation domain and incorporating an epitope of hemagglutinin. The clones were transfected into a strain of pEGY48 already containing lex-MDM2 (plex-202+PL containing full length MDM2) and pJK103. The top three p53 sequences shown in Fig. 6C. were derived from yeast obtained by colony screening, whereas the lower three were genetically engineered to contain the indicated fragments.

The resultant yeast colonies were examined for  $\beta$ -galactosidase activity in situ. Of approximately 5000 clones containing MDM2 fragments fused to the lexA DNA

binding domain, 17 were found to score positively in this assay. The clones could be placed into two classes. The first class (two clones) expressed low levels of  $\beta$ galactosidase (about 5-fold less than the other fifteen clones) and  $\beta$ -galactosidase expression was independent of p53 expression (Figure 6A). These two clones encoded MDM2 amino acids 190-340 and 269-379, respectively. The region shared between these two clones overlapped the only acidic domain in MDM2 (amino acids 230-301). This domain consisted of 37.5% aspartic and glutamic acid residues but no basic amino acids. This acidic domain appears to activate transcription only when isolated from the rest of the MDM2 sequence, because the entire MDM2 protein fused to lexA had no measurable  $\beta$ -galactosidase activity in the same assay (Table I, strain 3). The other class (15 clones) each contained the emino terminal region of MDM2 (Figure 6B). The  $\beta$ -galactosidase activity of these clones was dependent on p53 co-expression. To narrow down the region of interaction, we generated six additional clones by genetic engineering. The smallest tested region of MDM2 which could functionally interact with full length p53 contained MDM2 codons 1 to 118 (Figure 6B). The relatively large size of the domain required for interaction was consistent with the fact that when small sonicated fragments of MDM2 were used in the screening assay (200 bp instead of 600 bp average size), no positively scoring clones were obtained.

In a converse set of experiments, yeast clones containing fragments of p53 fused to the B42 AAD were screened for lexA-responsive reporter expression in the presence of a lexA-MDM2 fusion protein. Sequencing of the 14 clones obtained in the screen revealed that they could be divided into three subsets, one containing amino acids 1-41, a second containing amino acids 13-57, and a third containing amino acids 1-50 (Figure 2C). The minimal overlap between these three fragments contained codons 13-41. Although this minimal domain was apparently necessary for interaction with MDM2, it was insufficient, as the fragments required 9-12 amino acids on either side of codons 13-41 for activity (Figure 6C). To further test the idea that the amino terminal region of p53 was required for MDM2 binding, we generated an additional yeast strain expressing

the lexA-DNA binding domain fused to p53 codons 74-393) and the B42 acidic activation domain fused to full length MDM2. These strains failed to activate the same lexA-responsive reporter (Table I, strain 8), as expected if the N-terminus of p53 were required for the interaction.

TABLE I

STRAIN NUMBER	p53 CONSTRUCT	MDM2 CONSTRUCT	ACTIVATION
1	p53*	Vector <sup>b</sup>	-
2	p53°	lexA-MDM2b	+
3	Vector <sup>a</sup>	lexA-MDM2b	_
4	p53°	lexA-MDM2 (1-118) <sup>b</sup>	+
5	Vector <sup>a</sup>	lexA-MDM2 (1-118) <sup>b</sup>	_
6	B42-p53 (1-41) <sup>c</sup>	lexA-MDM2b	+
7	Ь42-р53 (1-41)°	Vector <sup>b</sup>	_
8,	lexA-p53 (74-393) <sup>b</sup>	B42-MDM2°	_
9	p53 (1-137)ª	lexA-MDM2b	-

The MDM2 and p53 proteins expressed in each strain, along with the relevant reporters, are indicated. Numbers in parentheses refer to the MDM2 or p53 amino acids encoded (absence of parentheses indicated full length protein, that is, MDM2 amino acids 1 to 491 or p53 amino acids 1 to 393). The lexaresponsive  $\beta$ -galactosidase reporter plasmid (pJK103, Kamens, J., et al., Mol. Cell. Biol. 10:2840-2847 (1990)) was present in all strains.

pRS314 vector (Nigro, J.M., et al., Mol. and Cell. Biol. 12:1357-1365 (1992).

\*plex(1-202)+PL vector, containing lexA DNA binding domain fused to insert (Ruden, D.M., et al., Nature 350:250-252 (1991).

pJG4-5 vector, containing B42 activation domain fused to insert.

 $^{4}(+)$  indicates that colonies turned blue following 24 hours of incubation on X-gal-containing selective medium, while (-) indicates that colonies remained white following 72 hours of incubation.

Sequence analysis showed that all p53 and MDM2 fragments noted in Figure 6 were ligated in frame and in the correct orientation relative to the B42 and lexA domains, respectively. Additionally, all clones compared in Figure 6 expressed the relevant proteins at similar levels, as shown by Western blotting (Figure 7).

The most striking results of these mapping experiments was that the region of p53 required to bind MDM2 was almost identical to the previously identified acidic activation domain of p53 (amino acids 20-42) (Unger, T., et al., EMBO J. 11:1383-1390 (1992); Miller, C.W., et al., Proc. Am. Assoc. Cancer Res. 33:386 (1992). This suggested that MDM2 inhibits p53-mediated transcriptional activation by "concealing" the activation domain of p53 from the transcriptional machinery. If this were true, the p53 activation domain, in isolation from the rest of the p53 protein, should still be inhibitable by full length MDM2. To test this hypothesis, we produced a hybrid protein containing the p53 activation domain (codons 1-73) fused to the lexA-DNA binding domain. This construct exhibited strong transcriptional activation of a lexA-responsive reporter (Figure 8), as predicted from previous experiments in which the p53 activation domain was fused to another DNA binding domain (Fields, S., et al., Science 249:1046-1049 (1990); Raycroft, L., et al., Science 249:1049-1051 (1990)). The lexA-p53 DNA construct was stably expressed in yeast along with the full length MDM2 expression vector (or the vector alone). MDM2 expression resulted in a five-fold decrease in reporter activity, demonstrating that MDM2 can specifically inhibit the function of the p53 activation domain regardless of the adjacent protein sequences tethering p53 to DNA (Figure 8).

METHODS. Strains were grown to mid-log phase in 2% dextrose before induction of p53 expression for 2 hours by the addition of 2% galactose. The lex-p53 construct was identical to lex-VP16 (YVlexA, Dalton, S., et al., Cell 68:597-612 (1992)) except that VP16 sequences were replaced by p53 sequences encoding amino acids 1 to 73.

The results obtained in the experiments discussed herein raise an interesting paradox. If MDM2 binds to (Figure 6) and conceals (Figure 8) the p53 activation

domain from the transcriptional machinery, how could the lexA-MDM2-p53 complex activate transcription from the lexA-responsive reporter (Table I, strain 2)? Because the only functional activation domain in the lexA-MDM2-p53 complex of strain 2 is expected to be contributed by p53, one might predict that it would be concealed by binding to MDM2 and thereby fail to activate. A potential resolution of this paradox is afforded by knowledge that p53 exists as a homotetramer (Stenger, J.E., et al., Mol. Carcinogenesis 5:102-106 (1992); Sturzbecher, H.W. et al., Oncogene 7:1513-1523 (1992). Thus the activation noted in the lexA-MDM2-p53 complex could be due to the presence of four individual activation domains contributed by the p53 tetramer, not all of which were concealed by MDM2. As a direct test of this issue, the domain of p53 required for homo-oligomerization (Stenger, J.E., et al., Mol. Carcinogenesis 5:102-106 (1992); Sturzbecher, H.W. et al., Oncogene 7:1513-1523 (1992) (the C-terminus) was removed from the p53 expression construct, so that it consisted of only codons 1-137. This truncated p53 polypeptide retained the entire activation domain (as shown in Figure 8, bar a) and the entire domain required for interaction with MDM2 (Table I, strain 6). Yet, when allowed to interact with lexA-MDM2, no transactivation of the lexAresponsive reporter was observed (Table I, strain 9). Because p53 did not inhibit lexA-MDM2 binding to the lexA reporter (Table I, strain 2), the result of strain 9 is likely to be due to a direct inhibition of the isolated p53 activation domain by MDM2.

#### Example 8

This example illustrates the production and characterization of antibodies specific for MDM2 epitopes.

The antigen preparations used to intraperitoneally immunize female (BALB/c X C57BL/5)F1 mice comprised bacterially expressed, glutathione-column purified glutathione-S-transferase-MDM2 (GST-MDM2) fusion protein. (One preparation was further purified on a polyacrylamide gel and electroeluted.) The fusion protein contains a 16 kD amino terminal portion of human MDM2 protein (amino acids 27 to

168). For immunization, the fusion protein was mixed with Ribi adjuvant (Ribi Immunochem Research, Inc.).

Two mice were sacrificed and their spleen cells fused to SP2/0s myeloma cells (McKenzie, et al., Oncogene, 4:543-548, 1989). Resulting hybridomas were screened by ELISA on trpE-MDM2 fusion protein-coated microtiter wells. The trpE-MDM2 fusion protein contains the same portion of MDM2 as the GST-MDM2 fusion protein. Antigen was coated at a concentration of 1  $\mu$ g/ml.

A second fusion was performed as described except hybridomas were screened on electroeluted, glutathione purified GST-MDM2. Positive hybridomas from both fusions were expanded and single cell subcloned. Subclones were tested by Western Blot for specificity to the 55 kD trpE-MDM2 and the 43 kD GST-MDM2 fusion proteins.

Two Western Blot positive subclones (1F2 and JG3) were put into mice for ascites generation. The resulting ascites were protein A purified. Both purified monoclonal antibodies tested positive by Western Blot and immunoprecipitation for the 90 kD migrating MDM2 protein present in a human osteosarcoma cell line (OsA-CL), which overexpresses MDM2, and negative in the HOS osteosarcoma, which does not overexpress MDM2.

ED9 was protein G-purified from ascites and found to be specific in cryostat immunohistochemistry for MDM2 in osteosarcoma cells, as was IF2.

Example 9

This example demonstrates the expression and detection of MDM2 at the cellular level.

To evaluate MDM2 expression at the cellular level, we produced monoclonal antibodies against bacterially generated fusion proteins containing residues 27 to 168 of MDM2. (See example 8.) Of several antibodies tested, mAb IF-2 was the most useful, as it detected MDM2 in several assays. For initial testing, we compared proteins derived

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from OsA-CL, a sarcoma cell line with MDM2 amplification but without p53 mutation (Table II) and proteins from SW480, a colorectal cancer cell line with p53 mutation (Barak et al., *EMBO 12*:461-468 (1993)) but without MDM2 amplification (data not shown). Figure 9 shows that the mAb IF-2 detected an intense 90 kd band plus several other bands of lower molecular weight in OsA-CL extracts, and a much less intense 90 kd band in SW480 extracts. We could not distinguish whether the low molecular weight bands in OsA-CL were due to protein degradation or alternative processing of MDM2 transcripts. The more than 20-fold difference in intensity between the signals observed in OsA-CL and SW480 is consistent with the greater than 20-fold difference in MDM2 gene copy number in these two lines. Conversely, the 53 kd signal detected with p53-specific mAb 1801 was much stronger in SW480 than in OsA-CL consistent with the presence of a mutated p53 in SW480 (Fig. 9).

Cells grown on cover slips were then used to assess the cellular localization of the MDM2 protein. A strong signal, exclusively nuclear, was observed in OsA-CL cells with the IF-2 mAb and a weaker signal, again strictly nuclear, was observed in SW480 (Fig. 10). The nuclear localization of MDM2 is consistent with previous studies of mouse cells (Barak et al., EMBO 12:461-468 (1993)) and the fact that human MDM2 contains a nuclear localization signal at residues 179 to 186. Reactivity with the p53-specific antibody was also confined to the nuclei of these two cell lines (Fig. 10), with the relative intensities consistent with the Western blot results (Fig. 9).

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The IF-2 mAb was then used (at 5  $\mu$ g/ml) to stain the seven primary sarcomas noted above. The nuclei of two of them (tumors #3 and #10) stained strongly (Fig. 11). Both of these tumors contained MDM2 gene amplification (Table II). In the five tumors without amplification, little or no MDM2 reactivity was observed (example in Fig. 11).

# TABLE II

	_				
TUMOR	TUMOR	TYPE	MDH TELONG	P53	OVER-
-	M-2	MEN	THE TOWN THE TOWN	ALTERATION	EXPRESSION
	3	Mrn	ABSENT	DELETION/ REARRANGEMENT	NONE
7	M-5	MFII	ABSENT	CGC-CUC MUTATION;	p53
3	M-7	MFH	PRESENT	MON COOK	
4	¥.	MEU		NONE OBSERVED	MDM2
		IJ LI	ABSENT	DELETION	NONE
6	M-14	MFH	ABSENT	NONE OBSERVED	6 2
9	M-15	MFH	ABSENT	Deremon	.I.v.
7	MIJA	nan		DELETTON	N.T.
	:	11.11	ABSENT	NONE OBSERVED	NONE
8	M-17	MFII	ABSENT	NONE OBGETTERS	
6	M-18	MFH	End SO K	NOME OBSERVED	N.T.
9			ABSENT	OVEREXPRESSED	p53
2	M-20	MFH	PRESENT	NONE OBSERVED	MDMO
=	L-5	LIPOSARCOMA	ABSENT	MOWN ORGAN	MUM2
1,	1			NONE OBSERVED	N.T.
3	L-1	LI POSARCOMA	ABSENT	AAC-AGC MUTATION;	N.T.
13	6-1	LIDOCADOMA		195-(239)-Ser	
	1	LAFUSARCUMA	PRESENT	NONE OBSERVED	8

# TABLE II (Cont.)

TUMOR         TVPE*         MAPLIF*           14         L-11         LIPOSARCOMA         ABSENT           15         KL5B         LIPOSARCOMA         ABSENT           16         KL7         LIPOSARCOMA         ABSENT           17         KL10         LIPOSARCOMA         ABSENT           19         KL11         LIPOSARCOMA         ABSENT           20         KL28         LIPOSARCOMA         PRESENT           21         KL30         LIPOSARCOMA         PRESENT           22         S189         LIPOSARCOMA         PRESENT           23         S131B         LIPOSARCOMA         PRESENT           24         OSA-CL         MFH         PRESENT						
C	TUMOR		TYPE.	MDM2 AMPLIFICATION <sup>b</sup>	P53 MITIATIONS	OVER.
KL5B LIPOSARCOMA  KL10 LIPOSARCOMA  KL11 LIPOSARCOMA  KL12 LIPOSARCOMA  KL28 LIPOSARCOMA  KL30 LIPOSARCOMA  KL30 LIPOSARCOMA  LIPOSARCOMA  KL30 LIPOSARCOMA  KL30 LIPOSARCOMA  KL30 LIPOSARCOMA  COSA-CL MFH	14	L-11	LIPOSARCOMA	ABSENT	MOTION:	EXPRESSION
KL5B LIPOSARCOMA KL10 LIPOSARCOMA KL11 LIPOSARCOMA KL12 LIPOSARCOMA KL28 LIPOSARCOMA KL30 LIPOSARCOMA G131B LIPOSARCOMA GOSA-CL MFH	1			***************************************	NONE OBSERVED	N.T.
KL7	15	KL5B	LIPOSARCOMA	ABSENT	CAG-UAG MUTATION;	N.T.
KL10   LIPOSARCOMA   KL11   LIPOSARCOMA   KL12   LIPOSARCOMA   KL28   LIPOSARCOMA   LIPOSARCOMA   KL30   LIPOSARCOMA   E131B   LIPOSARCOMA   E3131B   LIPOSARCOMA   E CSA-CL   MFH   F	16	KL7	LIPOSARCOMA	DDECENIM	dong (try)	
KL10   LIPOSARCOMA   KL11   LIPOSARCOMA   KL12   LIPOSARCOMA   KL28   LIPOSARCOMA   KL30   LIPOSARCOMA   S189   LIPOSARCOMA   S131B   LIPOSARCOMA   OSA-CL   MFH   I	;			TURRENT	NONE OBSERVED	N.T.
KL11   LIPOSARCOMA   KL12   LIPOSARCOMA   KL28   LIPOSARCOMA   KL30   LIPOSARCOMA   S189   LIPOSARCOMA   S131B   LIPOSARCOMA   OSA-CL   MFH   I	17	KL10	LIPOSARCOMA	ABSENT	NONE OBSERVED	£ 2
KL12       LIPOSARCOMA         KL28       LIPOSARCOMA         KL30       LIPOSARCOMA         S189       LIPOSARCOMA         S131B       LIPOSARCOMA         OSA-CL       MFH	18	KL11	LIPOSARCOMA	ABSENT	GGT-GAT MUTATION; EXON 5	N.T.
KL12   LIPOSARCOMA   KL28   LIPOSARCOMA   KL30   LIPOSARCOMA   S189   LIPOSARCOMA   S131B   LIPOSARCOMA   OSA-CL   MFH   I	,				STELL DONOR SITE	
KL28 LIPOSARCOMA KL30 LIPOSARCOMA S189 LIPOSARCOMA S131B LIPOSARCOMA OSA-CL MFH	19	KL12	LIPOSARCOMA	ABSENT	NONE OBSERVED	
KL30 LIPOSARCOMA S189 LIPOSARCOMA S131B LIPOSARCOMA OSA-CL MFH	20	VI 20				N.T.
KL30 LIPOSARCOMA S189 LIPOSARCOMA S131B LIPOSARCOMA OSA-CL MFH	7	NLCO		PRESENT	NONE OBSERVED	N.T.
S131B LIPOSARCOMA S131B LIPOSARCOMA OSA-CL MFH	21	KL30		PRESENT	NONE OBSERVED	8 2
S131B LIPOSARCOMA OSA-CL MFH	22	S189		DDFCFNT		N.I.
S131B LIPOSARCOMA OSA-CL MFH	]				NONE OBSERVED	N.T.
OSA-CL MFH	23	S131B	LIPOSARCOMA	ABSENT	NONE OBSERVED	
	24	OSA-CL	MFH	DODGONE		N.T.
				FRESENT	NONE OBSERVED	MDM2

MFH= malignant fibrous histiocytoma

<sup>b</sup> as assessed by Southern blot

• as assessed by Southern blot, sequencing of exons 5-8, or immunohistochemical analysis

d as assessed by immunohistochemical analysis; N.T. = not tested

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#### SEQUENCE LISTING

#### (1) GENERAL INFORMATION:

- (i) APPLICANT: BURRELL, MARILEE
  HILL, DAVID E.
  KINZLER, KENNETH W.
  VOGELSTEIN, BERT
- (ii) TITLE OF INVENTION: AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS
- (iii) NUMBER OF SEQUENCES: 5
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: BANNER, BIRCH, MCKIE AND BECKETT
  - (B) STREET: 1001 G STREET, N.W.
  - (C) CITY: WASHINGTON
  - (D) STATE: D.C.
  - (E) COUNTRY: USA
  - (F) ZIP: 20001
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

### (vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE: 07-APR-1993
- (C) CLASSIFICATION:

# (viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: KAGAN, SARAH A.
- (B) REGISTRATION NUMBER: 32,141
- (C) REFERENCE/DOCKET NUMBER: 01107.42798

# (ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 202-508-9100
- (B) TELEFAX: 202-508-9299
- (C) TELEX: 197430 BBMB UT

# (2) INFORMATION FOR SEQ ID NO:1:

# (1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 64 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

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- (v) FRAGMENT TYPE: N-terminal
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
- (viii) POSITION IN GENOME:
  - (A) CHROMOSOME/SEGMENT: 17q
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln

1 5 10 15

Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu Asn Asn Val Leu
20 25 30

Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Leu Ser Pro Asp

Asp Ile Glu Gln Trp Phe Thr Glu Asp Pro Gly Pro Asp Glu Ala Pro 50 55 60

- (2) INFORMATION FOR SEQ ID NO:2:
  - (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2372 base pairs

(B) TYPE: nucleic acid

(c) STRANDEDNESS: double	
(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: cDNA	
(iii) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: NO	
(vi) ORIGINAL SOURCE:	
(A) ORGANISM: Homo sapiens	
(H) CELL LINE: CaCo-2	
(viii) POSITION IN GENOME:	
(B) MAP POSITION: 12q12-14	
(ix) FEATURE:	
(A) NAME/KEY: CDS	
(B) LOCATION: 3121784	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:	
GCACCGCGCG AGCTTGGCTG CTTCTGGGGC CTGTGTGGCC CTGTGTGTCG GAAAGATGGA	60
GCAAGAAGCC GAGCCCGAGG GGCGGCCGCG ACCCCTCTGA CCGAGATCCT GCTGCTTTCG	120
CAGCCAGGAG CACCGTCCCT CCCCGGATTA GTGCGTACGA GCGCCCAGTG CCCTGGCCCG	180

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GAC	AGTO	GAA	TGAT	raca	CGA G	GCCC	'AGGG	C GI	CGTG	CTT	C CGC	CAGT	GTC	AGT	ccc	CGTG	240
AAG	GAAA	CTG	GGGZ	GTCI	TG A	.GGGA	.cccc	C GA	CTCC	AAGC	GCG	:AAA <i>I</i>	CCC	CGG2	ATGO	FTGA	300
																	300
GGA	GCAG	GCA			C AA												350
			Me	t Cy	rs As	n Th	r As	n Me	t Se	r Va	al Pı	co Ti	nr A	sp G	ly i	Ala	
				1				5				3	LO				
					ATT												398
Val			Ser	Gln	Ile	Pro	Ala	Ser	Glu	Glr	Gli	1 Thi	Let	ı Va	l Az	rg	
	15					20					25	;					
CCA	AAG	CCA	TTG	CTT	TTG	AAG	TTA	TTA	AAG	TCT	GTT	GGT	GCA	CAA	. AA	A	446
Pro	Lys	Pro	Leu	Leu	Leu	Lys	Leu	Leu	Lys	Ser	Val	Gly	Ala	Glr	ı Ly	78	
30					35					40					4	15	
GAC	ACT	TAT	ACT	ätg	AAA	GAG	GTT	CIT	TTT	TAT	CTT	GGC	CAG	TAT	AT.	r	494
qaA	Thr	Tyr	Thr	Met	Lys	Glu	Val	Leu	Phe	Tyr	Leu	Gly	Glr	тут	: I1	.e	
				50					55					60			
ATG	ACT	AAA	CGA	TTA	TAT	GAT	GAG	AAG	CAA	CAA	CAT	ATT	GTA	TAT	TGT	r	542
					Tyr												
			65					70					75		•		
rca	AAT	GAT	CTT	CTA	GGA	GAT	TTG	TTT	GGC	GTG	CCA	AGC	TTC	TCT	GTG	3	590
Ser	Asn	Asp	Leu	Leu	Gly	Asp	Leu	Phe	Gly	Val	Pro	Ser	Phe	Ser	· Va	1	
		80					85					80					

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AA	A GA	G CA	AGG	AAA	ATA	LAT	' ACC	ATC	ATC	TAC	AG	G AAC	TTO	G GT	A GT	A 638
Ly	s Glu	ı His	s Arg	Lys	Ile	туг	Th	r Mei	t Ile	₹ Ty:	r Ar	g As	n Le	u Va	l Va	1
	99					100					10					•
GT	CAA C	CAG	CAG	GAA	TCA	TCG	GAC	TCA	GGT	' ACA	TCT	GTG	AG7	C GAC	יממ ב	686
Va:	L Asr	Glr	ı Gln	Glu	Ser	Ser	Asp	Ser	Gly	Th:	Se	r Va	l Se	r Gl	n De	- 000 n
110	)				115					120				_ 01	12	
															12	<b>J</b>
AGO	TGI	CAC	CIT	GAA	GGT	GGG	AGT	GAT	CAA	AAG	GAC	CTT	' GTA	. מא	. GNG	
Arc	Cys	His	Leu	Glu	Gly	Gly	Ser	Asp	Gln	Lve	Ası	Lei	ı Va	ו מו	n Gl	734
				130					135		2		- • •	14		<b>u</b>
														11	U	
CII	' CAG	GAA	GAG	AAA	CCT	TCA	TCT	TCA	CAT	TTG	ىلملىك	ىلمانلى ،	מכת	CCN	m com	
Leu	Gln	Glu	Glu	Lys	Pro	Ser	Ser	Ser	His	Leu	Val	Sar	A	. CCA	. 101	782
			145					150				. Get			o sei	5
													155	•		
ACC	TCA	TCT	AGA	AGG	AGA	GCA	ATT	AGT	GAG	מרמ	GNN	("A A	220	mas		
Thr	Ser	Ser	Arg	Arg	Arg	Ala	Ile	Ser	Glu	Thr	GAA	GAA	AAT	TCA	GAT	830
		160					165		914	****	GIU			Ser	. Asp	)
												170				
GAA	TTA	TCT	GGT	GAA	CGA	CAA	AGA	ΔΔΑ	רפר	כאכ	222	mom	~~-			
Glu	Leu	Ser	Gly	<b>úl</b> u	Arg	Gln	Arg	Lve	Δτα	ui.	AAA T	1CT	GAT	AGT	ATT	878
	175				_	180		-, -	9	*11.0			Asp	Ser	Ile	!
											185					
TCC	CTT	TCC	TTT	GAT	GAA	AGC	CTG	сст	رسر:	TV-m	Om.					
Ser	Leu	Ser	Phe	qaA	Glu	Ser	Len	מכו	Lor	TGT.	GTA	ATA	AGG	GAG	ATA	926
190			•		195		⊂U	wia	reu		val	Ile	Arg	Glu	Ile	
										200					205	

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TGT	TG1	GAA	AGA	A AGO	AGT	AGC	AGI	GAA	TCI	' ACA	GGG	ACC	3 CC	A TC	:G 1	AAT	974
Cys	Суя	Glu	ı Arg	g Ser	Ser	Sei	s Sei	c Glu	ı Sei	Thi	G1;	y Th	r Pr	o Se	er'	Asn	
				210	)				215	5				22	20		
CCG	GAT	CTT	' GAT	GCT	GGT	GTA	AGT	' GAA	CAT	TCA	GGI	GAI	TG	G TT	GG	EAT	1022
Pro	Asp	Leu	Asp	Ala	Gly	Val	. Ser	Glu	His	Ser	Gl	y As <sub>l</sub>	p Tr	p Le	eu.	Asp	
			225					230					23			•	
CAG	GAT	TCA	GTT	TCA	GAT	CAG	TTT	AGT	GTA	GAA	TIT	GAA	GT.	r gaz	A T	CT	1070
Gln	Asp	Ser	Val	Ser	qaA	Gln	Phe	Ser	Val	Glu	Phe	e Glu	ı Va	l Gl	u s	Ser	
		240					245					250					
CTC	GAC	TCA	GAA	GAT	TAT	AGC	CTT	AGT	GAA	ĠAA	GGA	CAA	GAZ	CTC	СТ	CA	1118
Leu	Asp	Ser	Glu	Asp	Tyr	Ser	Leu	Ser	Glu	Glu	Gly	Glr	ı Gl	u Le	u S	Ser	
	255					260					265						
GAT	GAA	GAT	GAT	GAG	GTA	TAT	CAA	GTT	ACT	GTG	TAT	CAG	GCA	GGG	G	AG	1166
Asp	Glu	Asp	Asp	Glu	Val	Tyr	Gln	Val	Thr	Val	Tyr	Gln	Ala	a Gl	y G	Slu	
270					275					280				•		285	
AGT	GAT	ACA	GAT	TCA	TTT	GAA	GAA	GAT	CCT	GAA	ATT	TCC	TTA	GCT	' GJ	A.C	1214
Ser	Ąsp	Thr	Asp	Ser	Phe	Glu	Glu	Asp	Pro	Glu	Ile	Ser	Lei	ı Ala	a A	LSD	
				290					295					300		•	
TAT	TGG	AAA	TGC	ACT	TCA	TGC	TAA	GAA	ATG	AAT	ccc	CCC	CIT	CCA	TO	ZA.	1262
Tyr	Trp	Lys	Cys	Ξ'nΙ	Ser	Cys	Asn	Glu	Met	Asn	Pro	Pro	Leu	Pro	S	er	-302
			305					310					315			•	

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CA:	r TG	C AAC	C AGA	TG1	TGG	GCC	CTI	CG1	GAG	TAA	' TGG	CTT	CCI	GAZ	A GAT	1310
His	су:	s Asr	ı Arç	Cys	Trp	Ala	ı Leı	ı Arg	g Glu	ı Asr	ı Tr	p Lei	u Pro	 o Gl	u Ası	5
		320					325					330			•	•
AAZ	4 GGG	AAA	GAT	' AAA	GGG	GAA	ATC	TCT	' GAG	AAA	GCC	. AAA	CTG	GAA	AAC	1358
Lys	Gly	Lys	Asp	Lys	Gly	Glu	Ile	Ser	Glu	Lys	Ala	a Lys	Let	ı Glı	u Asr	1
	335	i				340	)				345	5				
		_														
TCA	ACA	. CAA	GCT	GAA	GAG	GGC	TTT	GAT	GTT	CCT	GAT	TGT	AAA	AAA	ACT	1406
		Gln	Ala	Glu	Glu	Gly	Phe	Asp	Val	Pro	Asp	Сув	Lys	Lys	s Thr	•
350					355					360					365	;
מיתמ	GTG	יחית ת	Cam	maa												
Tle	Val	AAI	NAT.	TCC	AGA	GAG	TCA	TGT	GTT	GAG	GAA	AAT	GAT	GAT	AAA	1454
	•42	voli	Asp	370	Arg	GIU	Ser	Cys		Glu	Glu	Asn	Asp	Asp	Lys	
				3 / 0					375					380	ı	
ATT	ACA	CAA	GCT	TCA	CAA	TCA	ממי	C22	3 Cm	~						
Ile	Thr	Gln	Ala	Ser	Gln	Ser	Gla	GAA.	AGT	GAA	GAC	TAT	TCT	CAG	CCA Pro	1502
			385				GIII	390	ser	GIU	qaA	Tyr		Gln	Pro	
	•							330					395			
TCA	ACT	TCT	AGT	AGC	ATT	ATT	TAT	AGC	AGC	CAA	C 2 2	~~m				
Ser	Thr	Ser	Ser	Ser	Ile	Ile	Tyr	Ser	Ser	Gln	GALA	OA1	41-1	AAA	GAG Glu	1550
		400					405			<b></b>	GIU	410	vai	гÀв	GIU	
												410				
TTT	GAA	AGG	GAA	GAA	ACC	CAA	GAC .	AAA	GAA	GAG .	agt	GTG	GAA	ىئىسابل	አርጥ	1500
Phe	Glu	Arg	Glu	Glu	Thr	Gln	Asp	Lys	Glu	Glu	Ser	Val	Glu	Ser	Ser	1598
	415					420					425					

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TTG	CCC	CTT	AAT	GCC	ATT	GAA	CCT	TGT	GTG	ATT	TGT	CAA	GGT	CGA	C	CT	1646
Leu	Pro	Leu	Asn	Ala	Ile	Glu	Pro	Cys	Val	Ile	Cys	Gln	Gly	y Arg	g P	ro	
430					435					440					4	45	
AAA	AAT	GGT	TGC	ATT	GTC	CAT	GGC	מממ	אכא	GGA	Cam	CTT	n ma	222			
						His											1694
		_	-	450			1	٠, ٥	455		1115	Leu	met			ys	
									433					460	)		
TTT	ACA	TGT	GCA	AAG	AAG	CTA	AAG	AAA	AGG	AAT	AAG	CCC	TGC	CCA	GT	ra	1742
						Leu											
			465					470					475				
TGT	AGA	CAA	CCA	TTA	CAA	ATG	ATT	GTG	CTA	ACT	TAT	TTC	CCC				1784
Cys	Arg	Gln	Pro	Ile	Gln	Met	Ile	Val	Leu	Thr	Tyr	Phe	Pro	<b>)</b>			
		480					485					490					
TAGI	TGAC	CT G	TCTA	TAAG	A GA	ATTA	ATATA	TTT	CTA	ACTA	TATA	ACC	TA (	GGAA:	I I	TAGA	1844
CAAC	CTGA	I AA	TTAT	TCAC	A TA	TATO	'AAAG	TGA	GAAA	ATG	CCTC	'AATI	CA (	CATAC	GAT	TTC	1904
TTCI	CTTT	'AG T	'ATA	TTGA	C CI	ACTT	TGGT	AGI	'GGAA	ATAG	TGAA	TACT	TA (	TAT	LAF	TTG	1964
ACTI	'GAAT	'AT G	TAGO	TCAT	C CI	TTAC	ACCA	ACT	CCTA	ATT	TTAA	ATAA	TT 1	CTAC	CTC	TGT	2024
L'I'IA	AATG	AG A	AGTA	.CTTG	G TT	TITT	TITT	CTI	'AAAT	ATG	TATA	TGAC	AT I	TAAZ	ATG	TAA	2084
لا ملحلت	ת הידי איני			a. a.													
	LIMI	11 I	1 1 T.T.	ADAD	C CG	AGTC	TTGC	TCT	GTTA	CCC .	AGGC	TGGA	GT G	CAGI	rgg	GTG	2144
<b>ئىلى</b>	TGCC	מ ידר	ساتات	א א כי כי	ш ст.	-	T022	<b></b>		·							2204
		^				GCCC.	7000	CGG	GITC	GCA	CCAT	TCTC	CT G	درسار	־מכ	ירידי	2204

- 42 -

2264

2324

2372

CCCAATTAGC TIGGCCTACA GTCATCTGCC ACCACACCTG GCTAATTTTT TGTACTTTTA
GTAGAGACAG GGTTTCACCG TGTTAGCCAG GATGGTCTCG ATCTCCTGAC CTCGTGATCC
GCCCACCTCG GCCTCCCAAA GTGCTGGGAT TACAGGCATG AGCCACCG
(2) INFORMATION FOR SEQ ID NO:3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 491 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
Met Cys Asn Thr Asn Met Ser Val Pro Thr Asp Gly Ala Val Thr Thr
1 5 10 15

Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro
20 25 30

Leu Leu Lys Leu Lys Ser Val Gly Ala Gln Lys Asp Thr Tyr
35 40 45

Thr Met Lys Glu Val Leu Phe Tyr Leu Gly Gln Tyr Ile Met Thr Lys
50 55 60

Arg		и Ту:	r Ası	Glı	1 Lys		ı Glr	ı His	∃ Il∈	• Val		Cys	Ser	Asn	Asp
Let	ı Lei	ı Gl	/ Asp	) Leu	ı Phe	Gly	. Val	. Pro	Ser			· Val	Lvs	Glu	His
				85					90					95	******
Arg	Lys	; Il∈	100		Met	Ile	Tyr	Arg		Leu	Val	Val	Val	Asn	Gln
Gln	. Glu			Asp	Ser	Gly	Thr	Ser	Val	Ser	Glu	Asn	Arg	Cys	His
<b>T</b>	<b>a</b> 1	115					120					125			
Leu	130		GIA	Ser	qaA	Gln 135	Lys	Asp	Leu	Val	Gln 140	Glu	Leu	Gln	Glu
Glu 145	Lys	Pro	Ser	Ser	Ser	His	Leu	Val	Ser	Arg 155	Pro	Ser	Thr	Ser	
Arg	Arg	Arg	Ala	Ile	Ser	Glu	Thr	Glu	Glu		Ser	qaA	Glu	Leu	160 Ser
				165					170					175	
Gly	Glu	Arg	Gln 180	Arg	Lys	Arg	His	Lys 185	Ser	Asp	Ser	Ile	Ser 190	Leu	Ser
Phe	Asp	Glu 195	Ser	Leu	Ala		Cys 200	Val	Ile	Arg		Ile 205	Cys	Cys	Glu
Arg	Ser	Ser	Ser	Ser	Glu	Ser	Thr	Gly	Thr	Pro		Asn .	Pro 2	Asp :	Leu
	210					215					220				

- 44 -

Ası	Ala	a Gl	y Va	l Se	Glu	His	Sei	Gly	/ Asp	Trp	Leu	ı Asp	Gln	. Asp	Ser
225	5				230					235					240
Va]	l Sei	: As	p Gl			Val	Glu	ı Phe	Glu	Val	Glu	Ser	Leu	Asp	Ser
				245	5				250	١				255	
Glu	λετ	, The r	× Co.	<b>.</b>											
-		, <u>-</u> y	26	r Leu n	ser	Glu	Glu			Glu	Leu	Ser	Asp	Glu	Asp
			20	J				265					270		
Asp	Glu	. Va:	l Ty:	r Gln	Val	Thr	Va 1	We say	· (15	21-	<b>6</b> 1.		_		
		27					280		GIII	Ala	GIY		Ser	qaA	Thr
												285			
Asp	Ser	Phe	e Glu	ı Glu	Asp	Pro	Glu	Ile	Ser	Leu	Ala	Asp	Tur	T	Tara
	290					295					300		-1-	TTP	пув
Cys	Thr	Ser	Сув	Asn	Glu	Met	Asn	Pro	Pro	Leu	Pro	Ser	His	Cys	Asn
305					310					315					320
Arg	Cys	Trp	Ala	Leu	Arg	Glu	Asn	Trp	Leu	Pro	Glu	qaA	Lys	Gly	Lys
				325					330					335	
7	T	<b>63</b> -													
Asp	губ	GIY		Ile	Ser	Glu	Lys	Ala	Lys	Leu	Glu	Asn	Ser	Thr	Gln
			340					345					350		
Ala	Glu	Glu	Glv	Dhe	7 00	77n 7	D	_	_						
		355	Cly	Phe	web	Val		Asp	Cys	Lys	Lys	Thr	Ile	Val	Asn
							360					365			
Asp	Ser	Arg	Glu	Ser	Cys	Va1	Glıı	Glu	755	7	7 m	T			
	370	•				375			<b>-1011</b>			тÀв	TIE ,	Thr	Gln
						-					380				

- 45 -

Ala Ser Gln Ser Gln Glu Ser Glu Asp Tyr Ser Gln Pro Ser Thr Ser 385

Ser Ser Ile Ile Tyr Ser Ser Gln Glu Asp Val Lys Glu Phe Glu Arg

Glu Glu Thr Gln Asp Lys Glu Glu Ser Val Glu Ser Ser Leu Pro Leu
420 425 430

Asn Ala Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro Lys Asn Gly
435
440
445

Cys Ile Val His Gly Lys Thr Gly His Leu Met Ala Cys Phe Thr Cys
450 455 460

Ala Lys Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val Cys Arg Gln
465 470 475 480

Pro Ile Gln Met Ile Val Leu Thr Tyr Phe Pro
485 490

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1710 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: NO	
(Vi) ORIGINAL SOURCE:  (A) ORGANISM: Mus musculus	
(ix) FEATURE:	
(A) NAME/KEY: CDS	
(B) LOCATION: 2021668	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:	
52g 1D NO:4:	
GAGGAGCCGC CGCCTTCTCG TCGCTCGAGC TCTGGACGAC CATGGTCGCT CAGGCCCCGT	60
CCGCGGGGCC TCCGCGCTCC CCGTGAAGGG TCGGAAGATG CGCGGGAAGT AGCAGCCGTC	120
TGCTGGGCGA GCGGGAGACC GACCGGACAC CCCTGGGGGGA CCCTCTCGGA TCACCGCGCT	180
TCTCCTGCGG CCTCCAGGCC A ATG TGC AAT ACC AAC ATG TCT GTG TCT ACC	231
Met Cys Asn Thr Asn Met Ser Val Ser Thr	
1 5 10	
GAG GGT GGT GGA AGG AGG	
GAG GGT GCT GCA AGC ACC TCA CAG ATT CCA GCT TCG GAA CAA GAG ACT	279
Glu Gly Ala Ala Ser Thr Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr	
15 20 25	

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CTG	GTT	AGA	CCA	AAA	CCA	TTG	CTT	TTG	AAG	TTG	TTA	AAG	TCC	GTT	' GG#	4	327
							. Leu										
			30					35					4 (			_	
GCG	CAA	AAC	GAC	ACT	TAC	ACT	ATG	AAA	GAG	ATT	ATA	TTT	TAT	ATT	GGC	:	375
							Met										
		45					50					55					
							TTA										423
Gln	Tyr	Ile	Met	Thr	Lys	Arg	Leu	Tyr	Asp	Glu	Lys	Gln	Gln	His	Ile	<b>e</b>	
	60					65					70						
GTG	TAT	TGT	TCA	AAT	GAT	CTC	CTA	GGA	GAT	GTG	TTT	GGA	GTC	CCG	AGT	4	171
	Tyr	Cys	Ser	naA	Asp	Leu	Leu	Gly	Asp	Val	Phe	Gly	Val	Pro	Ser	<b>:</b>	
75					80					85					90	)	
TTC	TCT	GTG	AAG -	GAG	CAC	AGG	AAA	ATA	TAT	GCA	ATG	ATC	TAC	AGA	AAT	5	519
Pne	Ser	Val	Lys		His	Arg	Lys	Ile	Tyr	Ala	Met	Ile	Tyr	Arg	Asr	ı	
				95					100					105			
עידייד	CTC	~~ <del>~</del>	Cm.														
							GAC										67
<u> Leu</u>	VQ1	AIG	110	ser	GIU	Gin	Asp		Gly	Thr	Ser	Leu	Ser	Glu	Ser	•	
			110					115					120				
AGA	ССТ	ር <u>ኦ</u> ሮ	درئ	כא <i>א</i>	CCm	ccc	<b>3</b> C.T.	<b>~</b> -									
Ara	Ara	Gln	Dro	GAA	GGT.	C1	AGT	GAT	CTG -	AAG	GAT	CCT	TTG	CAA	GCG	6	15
-3		125	-10	GIU	gry	отА	Ser	qaA	Leu	Lys	Asp		Leu	Gln	Ala	,	
							130					135					

- 48 -

CC	A CC	A GA	A GAG	AAA G	CCI	TCA	TCI	TCI	GAT	TT	TTA A	TCT	AGA	ىس	2 m/		~ ~
Pr	o Pro	o Gl	ı Glı	ı :ye	Pro	Ser	Ser	Se	r Ası	) Le	u Ile	e Ser	· Arc	T.A	J I	· · ·	66:
	14					145					150			, 110	u s	er	
AC	TC	TCI	AGA	AGG	AGA	TCC	ATT	AGT	' GAG	ACA	A GAA	GAG	אאר	אריא	\ C7	\m	
The	Sei	Ser	Arg	Arg	Arg	Ser	Ile	Ser	Glu	ı Th:	r Glu	Glu	Agn	ישטאל.	~ X		711
155	i				160					165						50 70	
															1	70	
GAG	CTA	CCI	' GGG	GAG	CGG	CAC	CGG	AAG	CGC	CGC	AGG	TCC	رسرت	TOO	· 1784		
Glu	Leu	Pro	Gly	Glu	Arg	His	Arg	Lys	Arg	Arc	Aro	Ser	T.A11	Sas	~ DI		759
				175					180		, 3	-	200	185		.16	
														101	•		
GAT	CCG	AGC	CTG	GGT	CTG	TGT	GAG	CTG	AGG	GAG	ATG	TGC .	<b>AGC</b>	ccc	cc	C	
Asp	Pro	Ser	Leu	Gly	Leu	Cys	Glu	Leu	Arg	Glu	Met	Cys	Ser	GGC.	. GI	•••	807
			190					195				-7-2	200	GLY	Gı	-У	
													200				
ACG	AGC	AGC	AGT	AGC	AGC	AGC	AGC	AGC	GAG	TCC	ACA	GAG 2	ACG	ררר	ሞርረ	<b>-</b>	055
Thr	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Glu	Ser	Thr	Glu	Thr	Dro		<b>3</b>	855
		205					210					215		110	36	I.	
CAT	CAG	GAT	CTT	GAC	GAT	GGC	GTA	AGT	GAG	CAT	TCT	GGT (	። ግልጥ ነ	זיכר	مسد		
His	Gln	Asp	Leu	Asp	qaA	Gly	Val	Ser	Glu	His	Ser	Gly	Δen	ريور	Ta		903
	220					225					230	<b></b> ,	p	Cys	TE	u	
GAT	CAG	GAT	TCA	GTT '	TCT	GAT	CAG '	TTT .	AGC	GTG	GAA '	TTT G	י ממ:	بلعلات	GNO		0.77
Asp	Gln	qaA	Ser	Val	Ser	Asp	Gln	Phe	Ser	Val	Glu	Phe (	( G7:1	 7757	GAU.		951
235					240					245				AGT			
															250	J	

- 49 -

TCT	CTG	GAC	TCG	GAA	GAT	TAC	AGC	CTG	AGT	' GAC	GAA	GGG	CAC	GAG	CTC	999
Ser	Leu	Asp	Ser	Glu	Asp	Tyr	Ser	Leu	Sei	: Ası	o Glu	ı Gly	/ His	Glu	Leu	333
				255					260					265		
TCA	GAT	GAG	GAT	GAT	GAG	GTC	TAT	CGG	GTC	ACA	GTC	TAT	CAG	ACA	GGA	1047
Ser	Asp	Glu	Asp	qaA	Glu	Val	Tyr	Arg	Val	Thr	. Val	. Tyr	Gln	Thr	Gly	
			270					275					280			
GAA	AGC	GAT	ACA	GAC	TCT	TTT	GAA	GGA	GAT	CCT	GAG	ATT	TCC	TTA	GCT	1095
Glu	Ser	Asp	Thr	Asp	Ser	Phe	Glu	Gly	Asp	Pro	Glu	Ile	Ser	Leu	Ala	
		285					290					295				
GAC	TAT	TGG	AAG	TGT	ACC	TCA	TGC	AAT	GAA	ATG	AAT	CCT	CCC	CTT	CCA	1143
Asp		Trp	Lys	Cys	Thr	Ser	Cys	Asn	Glu	Met	Asn	Pro	Pro	Leu	Pro	
	300					305					310					
דירי <i>א</i>	C N C	maa														
I CA	CAC,	TGC	AAA	AGA	TGC	TGG	ACC	CTT	CGT	GAG	AAC	TGG	CTT	CCA (	GAC	1191
315	nis	cys	rys	Arg		Trp	Thr	Leu	Arg	Glu	Asn	Trp	Leu	Pro	Asp	
3 1 3					320					325					330	
ידענ	מממ	GGG	מממ	Cam												
Asp.	ive	Glv	Lic	GAT .	AAA	GTG	GAA	ATC	TCT	GAA	AAA	GCC	AAA (	CTG (	AAE	1239
.op	_ys	GIY	пуs	335	rys	Val	Glu	Ile		Glu	Lys	Ala	Lys	Leu	Glu	
				233					340					345		
AC :	rca :	GCT	CAG	GCD (	ממם	CAA .	ccc .			_						
sn :	Ser	Ala	Gln	Ala	GI.	GAA (	GGC !	rrg (	SAT (	GTG	CCT	GAT (	GGC I	AAA A	\AG	1287
	- <b>-</b>		350	-144	JIU	GIU		_	Asp	Val	Pro	qaA	Gly	Lys	Lys	
								355					360			

- 50 -

CTO	ACA	GAC	AA?	GAI	GCI	AAA	GAG	CCZ	A TGI	GCI	GAG	GAC	GA(	AG(	C GA	.G	1335
Lei	ı Thr	Glu	ı Asr	ı Asp	Ala	a Lys	Glu	ı Pro	э Суя	s Ala	a Glı	u Gl	u As	p Se	r G	lu	
		365					370					37					
GAG	AAG	GCC	GAA	CAG	ACG	CCC	CTG	TCC	CAG	GAG	AGT	GAC	GAC	TAT	TC	C.	1383
Glu	Lys	Ala	Glu	Gln	Thr	Pro	Leu	Ser	Glr	ı Glu	ı Seı	: Asj	p As	р Ту	r Se	er	
	380					385					390						
CAA	CCA	TCG	ACT	TCC	AGC	AGC	ATT	GTT	TAT	AGC	AGC	CAA	GAA	AGC	GTO	3	1431
Gln	Pro	Ser	Thr	Ser	Ser	Ser	Ile	Val	. Tyr	Ser	Ser	Glr	ı Glı	ı Se:	r Va	1	
395					400					405					41		
AAA	GAG	TTG	AAG	GAG	GAA	ACG	CAG	CAC	AAA	GAC	GAG	AGT	GTG	GAA	TCI		1479
Lys	Glu	Leu	Lys	Glu	Glu	Thr	Gln	His	Lys	Asp	Glu	Ser	Val	. Gli	ı Se	r	
				415					420					425	5		
AGC	TTC	TCC	CTG	AAT	GCC	ATC	GAA	CCA	TGT	GTG	ATC	TGC	CAG	GGG	CGG	}	1527
Ser	Phe	Ser	Leu	Asn	Ala	Ile	Glu	Pro	Cys	Val	Ile	Cys	Gln	Gly	Ar	g	
			430					435					440				
CCT	AAA	TAA	GGC	TGC	ATT	GTT	CAC	GGC	AAG	ACT	GGA	CAC	CTC	ATG	TCA		1575
Pro	Lys	Asn	Gly	Cys	Ile	Val	His	Gly	Lys	Thr	Gly	His	Leu	Met	Ser	r	
		445					450					455					
rgt	TTC	ACG	TGT	GCA	AAG	AAG	CTA .	AAA	AAA	AGA .	AAC	AAG	CCC	TGC	CCA		1623
.ys		Thr	Сув	Ala	Lys	Lys	Leu	Lys	Lys	Arg	Asn	Lys	Pro	Сув	Pro		
	460					465					470						

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GTG TGC AGA CAG CCA ATC CAA ATG ATT GTG CTA AGT TAC TTC AAC

Val Cys Arg Gln Pro Ile Gln Met Ile Val Leu Ser Tyr Phe Asn

485

TAGCTGACCT GCTCACAAAA ATAGAATTTT ATATTTCTAA CT

1710

- (2) INFORMATION FOR SEQ ID NO:5:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 489 amino acids
    - (B) TYPE: amino acid
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: protein
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Cys Asn Thr Asn Met Ser Val Ser Thr Glu Gly Ala Ala Ser Thr 1 5 10 15

Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro
20 25 30

Leu Leu Lys Leu Lys Ser Val Gly Ala Gln Asn Asp Thr Tyr
35 40 45

Thr Met Lys Glu Ile Ile Phe Tyr Ile Gly Gln Tyr Ile Met Thr Lys
50 55 60

210

Ar	g 5	Leu	Ту	r As	p Gl	u Ly 7		n Gli	ı His	5 Il€	∍ Va] 75		Сув	S Se:	r Ası	n Asp 80
Lei	u	Leu	Gl	y As			e Gly	/ Val	Pro	Ser	Phe	Ser	. Val	. Lys	s Glu	ı His
					8	5				90	)				95	5 .
Arg	<b>3</b> :	Lys	Ile			a Met	: Ile	тут	Arg	Asn	Leu	Val	Ala	Val	. Ser	Gln
				10	U				105					110	)	
Gln	1 2	Asp			y Thi	s Ser	Leu	Ser	Glu	Ser	Arg	Arg	Gln	Pro	Glu	Gly
			115	•				120					125			
Gly		Ser	Asp	Let	ı Lye	gaA s	Pro	Leu	Gln	Ala	Pro	Pro	Glu	Glu	Lys	Pro
	1	L30					135					140				
Ser	S	Ser	Ser	Asp	Leu	Ile	Ser	Arg	Leu	Ser	Thr	Ser	Ser	Arg	Arg	Arg
145		•				150					155					160
Ser	I	le	Ser	Glu	Thr	Glu	Ğlu	Asn	Thr	Asp	Glu	Leu	Pro	Gly	Glu	Arq
					165					170					175	
His	A	rg	Lys		Arg	Arg	Ser	Leu	Ser	Phe	qaA	Pro	Ser	Leu	Gly	Leu
				180					185					190		
Ore	c	1,,	۲	2	~1											
CyB	-		195	Arg	GIU	Met.	Сув		Gly	Gly	Thr	Ser	Ser	Ser	Ser	Ser
		•						200					205			
Ser	Se	er S	Ser	Glu	Ser	Thr	Glu	Thr	Pro	Ser	His (	Gln .	Asp :	Leu	Asp	Asp

215

220

- 53 -

Gl	y Vai	l Se:	r Glı	u Eis	5 Set	- G1,	. Ac-	Carr	<b>.</b> .						Ser
225					230		, wer	CAR	. Let			Asp	Ser	· Val	Ser
	-				230	,		<b>.</b> ;		235					240
7 ~~	- 01-	- 51-				•			-						
ASI	GII	ı Phe	e Sei	r Val	l Glu	ı Phe	Glu	Val	Glu	Ser	Leu	Asp	Ser	Glu	Asp
				245	5				250	ı				255	
Туг	Ser	Lei	ı Ser	Asp	Glu	Gly	His	Glu	Leu	Ser	Asp	Glu	Asp	Asp	Glu
			260					265					270		
Val	Tyr	Arc	y Val	Thr	Val	Tyr	Gln	Thr	Gly	Glu	Ser	Asp	Thr	Asp	Ser
		275					280					285			-
Phe	Glu	Gly	' Asp	Pro	Glu	Ile	Ser	Leu	Ala	Asn	Тч	m	T	<b>G</b>	~
	290					295				Agp		ιτρ	гув	Cys	Thr
											300				
Ser	Cys	Asn	Glu	Met	Aen	Pro	Dro	T		_					
305					Asn	110	PIO	neu	Pro		His	Сув	Lys	Arg	Cys
	•				310					315					320
سد <sub>ىك</sub>	Th~	T a	7	<b>~</b> 3	_	_									
110	1111	Leu	Arg		Asn	Trp	Leu	Pro	Asp	qaA	Lys	Gly	Lys	qaA	Lys
				325					330					335	
Val	Glu	Ile	Ser	Glu	Lys	Ala	Lys	Leu	Glu	naA	Ser	Ala	Gln	Ala	Glu
			340					345					350		
Glu	Gly	Leu	Asp	Val	Pro	qaA	Gly	Lys	Lys	Leu	Thr	Glu	Asn	qaA	Ala
		355					360					365		-	
Lys	Glu	Pro	Cys	∴la	Glu	Glu	qaA	Ser	Glu	Glu	Lve	Ala	Glu	G] n	Th~
	370					375					380			2111	TIIL
						_					200				

- 54 -

Pro Leu Ser Gln Glu Ser Asp Asp Tyr Ser Gln Pro Ser Thr Ser Ser 385

Ser Ile Val Tyr Ser Ser Gln Glu Ser Val Lys Glu Leu Lys Glu Glu
405 410 415

Thr Gln His Lys Asp Glu Ser Val Glu Ser Ser Phe Ser Leu Asn Ala

Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro Lys Asn Gly Cys Ile
435 440 445

Val His Gly Lys Thr Gly His Leu Met Ser Cys Phe Thr Cys Ala Lys
450
450

Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val Cys Arg Gln Pro Ile 465

Gln Met Ile Val Leu Ser Tyr Phe Asn 485 -55-International Application No: PCT/

1

MICROORG	ANISMS
Optional Sheet in connection with the microorganism referred to on p	nege10, line19 of the description 4
A. IDENTIFICATION OF DEPOSIT 1	
Further deposits are identified on an additional sheet 1 2	
Name of depositary institution *	
AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country)	12301 Parklawn Drive Rockville, Maryland 20852 United States of America
Date of deposit 6	Accession Number •
March 11, 1993	HB 11290
8. ADDITIONAL INDICATIONS! (leave blank if not applicable).	This information is continued on a separate attached eheet
Hybridoma: IF2 In respect to those designati is sought a sample of the deposite available until the publication of European patent or until the date refused or withdrawn or is deemed of such a sample to an expert nomi the sample. (Rule 28(4) EPC)  C. DESIGNATED STATES FOR WHICH INDICATIONS ARE	the mention of the grant of the on which the application has been to be withdrawn, only the issue nated by the person requesting
D. SEPARATE FURNISHING OF INDICATIONS (lears blank The indications listed below will be submitted to the international "Accession Number of Deposit")	
E. This sheet was received with the international application where the state of th	M. DYMS (Avihorized Officer)
was	(Authorized Officer)

ANNEX M3

International	Application	No: PCT/
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	MICROORGANISMS
Optional Sheet in connection with the microorganism	m relarred to on page 10 , line 19 of the description t
A. IDENTIFICATION OF DEPOSIT	of the description -
Further deposits are identified on an additional si	heet a
Name of depositary institution 4	
AMERICAN TYPE CULTURE COLL	
Address of depositary institution (including postal co	ode and country) 4
12301 Parklawn Drive Rockville, Maryland 20852,	USA
Date of deposit 4	Accession Number 4
March 11, 1993	HB 11290
S. ADDITIONAL INDICATIONS ! (leave blank if	not applicable). This information is continued on a separate attached sheet
IF2 - Hybridoma	
5, 5, 7, 25, 112	
C. DESIGNATED STATES FOR WATER INC.	
	ATIONS ARE MADE 1 (if the indications are not for all designated States)
•	
D. SEPARATE FURNISHING OF INDICATION	\$ * (leave blank if not applicable)
"Accession Number of Deposit")	is International Bureau later * (Specify the general nature of the Indications e.g.,
• 🗇 -	
E. This sheet was received with the international	application when filed (to be checked by the receiving Office)
	M. Wilmes (Authorized Officer)
The date of receipt (from the applicant) by the	) International Bureau 14
was	
	(Authorized Officer)

Form PCT/RO/134 (January 1981)

.1	nternational Application No: PCT/ /
MICROOR	GANISMS
Optional Sheet in connection with the microorganism referred to or	10 How 19 of the description t
A. IDENTIFICATION OF DEPOSIT	
Further deposits are identified on an additional sheet	
Name of depositary inetitution 4	
AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country 12301 Parklawn Drive Rockville, Maryland 20852, USA	), ◆
Date of deposit *	Accession Number 4
March 11, 1993	HB 11291
B. ADDITIONAL INDICATIONS ! (leave blank if not applicable	a). This information is continued on a separate attached sheet
ED9 - Hybridoma	
C. DESIGNATED STATES FOR WHICH INDICATIONS AR	E MADE ! (If the indications are not for all designated States)
•	
D. SEPARATE FURNISHING OF INDICATIONS (leave big	nh if not applicable
"Accession Number of Deposit")	al Bureau later • (Specify the general nature of the indications e.g.,
E This sheet was received with the international application	(Authorized Officer)
The date of receipt (from the applicant) by the Internation.	si Bureau 19
	(Authorized Officer)

## **CLAIMS**

- 1. A method of diagnosing a neoplastic tissue in a human comprising:

  detecting amplification of human MDM2 gene or elevated expression of a
  human MDM2 gene product in a tissue or body fluid isolated from a human, wherein
  amplification of the human MDM2 gene or elevated expression of human MDM2 gene
  product provides a diagnosis of neoplasia or the potential for neoplastic development.
  - The method of claim 1 wherein gene amplification is detected.
- 3. The method of claim 1 wherein elevated expression of a gene product is detected, said gene product being mRNA.
- 4. The method of claim 1 wherein elevated expression of a gene product is detected, said gene product being human MDM2 protein.
- 5. The method of claim 3 wherein said mRNA is detected by Northern blot analysis by hybridizing mRNA from said tissue to a human MDM2 nucleotide probe.
- 6. The method of claim 5 wherein the human MDM2 nucleotide probe comprises nucleotides 1-2372 of human MDM2, as shown in Figure 1, or fragments thereof consisting of at least 14 contiguous nucleotides.
- 7. The method of claim 4 wherein human MDM2 protein is detected by Western Blot analysis by reacting human MDM2 proteins with antibodies which are immunospecific for MDM2 protein.
- 8. The method of claim 2 wherein the gene amplification is detected using polymerase chain reaction.
- 9. The method of claim 2 wherein amplification of the human MDM2 gene is detected by Southern blot analysis wherein the human MDM2 gene is hybridized with a nucleotide probe which is complementary to hMDM2 DNA.
- 10. The method of claim 2 wherein gene amplification is determined by comparing the copy number of hMDM2 in the tissue to the copy number of hMDM2 in a normal tissue of the human.

- 11. The method of claim 3 wherein elevated expression of mRNA is determined by comparing the amount of hMDM2 mRNA in the tissue to the amount of hMDM2 mRNA in a normal tissue of the human.
- 12. The method of claim 4 wherein elevated expression of hMDM2 protein is determined by comparing the amount of hMDM2 protein in the tissue to the amount of hMDM2 protein in a normal tissue of the human.
- 13. The method of claim 2 wherein gene amplification is detected when at least 3-fold more hMDM-2 DNA is observed in the tissue relative to a control sample comprising a normal tissue.
- 14. The method of claim 3 wherein elevated expression is detected when at least 3-fold more hMDM-2 mRNA is observed in the tissue relative to a control sample comprising a normal tissue.
- 15. The method of claim 4 wherein elevated expression is detected when at least 3-fold more hMDM2 protein is observed in the tissue relative to a control sample comprising a normal tissue.
  - 16. The method of claim 1 wherein the neoplasia is a sarcoma.
- 17. The method of claim 16 wherein the sarcoma is a liposarcoma, malignant fibrous histiocytoma, or osteosarcoma.
- 18. A cDNA molecule comprising nucleotides 1 to 2372, as shown in Figure 1, or fragments thereof, consisting of at least 14 contiguous nucleotides.
- 19. The cDNA molecule of claim 18 comprising the coding sequence of human MDM2.
  - 20. Human MDM2 protein substantially free of other human proteins.
- 21. A preparation of antibodies specifically immunoreactive with human MDM2 protein.
  - 22. The preparation of claim 21 wherein the antibodies are monoclonal antibodies.
- 23. A nucleotide probe comprising a sequence of at least 10 nucleotides which are complementary to nucleotides 1-2372 of human MDM2 gene, as shown in Figure 1.

- 24. A kit for detecting the amplification of a human MDM2 gene in a human tissue or body fluid sample comprising: a nucleic acid probe capable of hybridizing to said human MDM2 gene under conditions of high stringency, and instructions for determining said amplification.
- 25. A kit for detecting elevated expression of a human MDM2 mRNA in a human tissue or body fluid sample comprising: a nucleic acid probe capable of hybridizing to said mRNA, and written instructions for determining elevated expression of mRNA.
- 26. A kit for detecting elevated expression of a human MDM2 protein in a human tissue or body fluid sample comprising MDM2 protein-specific antibodies and written instructions for determining elevated expression of human MDM2 protein.
- 27. A method of treating a neoplastic cell or a cell having neoplastic potential, comprising:

administering to a cell a therapeutically effective amount of an inhibitory compound which interferes with the expression of human MDM2 gene.

- 28. The method of claim 27 wherein expression of the human MDM2 gene is inhibited by administering antisense oligonucleotides.
- 29. The method of claim 27 wherein expression of the human MDM2 gene is inhibited by administering triple-strand forming oligonucleotides which interact with DNA.
- 30. A method for identifying compounds which interfere with the binding of human MDM-2 to human p53, comprising:

binding a predetermined quantity of a first human protein which is detectably labelled to a second human protein;

adding a compound to be tested for its capacity to inhibit binding of said first and second proteins to each other;

determining the quantity of the first human protein which is displaced from or prevented from binding to the second human protein;

wherein the first human protein is MDM-2 and the second human protein is p53 or the first human protein is p53 and the second human protein is MDM-2.

- 31. The method of claim 30 wherein one of said two human proteins is fixed to a solid support.
- 32. The method of claim 30 wherein an antibody specifically immunoreactive with said second human protein is used to separate first human protein bound from unbound first human protein.
- 33. A method for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification, comprising:

administering a polypeptide to tumor cells which contain a human MDM2 gene amplification, said polyptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide being capable of binding to human MDM2.

- 34. The method of claim 33 wherein said polypeptide comprises amino acids 1-41 of p53.
- 35. The method of claim 33 wherein said polypeptide comprises amino acids 13-57 of p53.
- 36. The method of claim 33 wherein said polypeptide comprises amino acids 1-50 of p53.
- 37. A method for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification, comprising:

administering to tumor cells which contain a human MDM2 gene amplification a DNA molecule which expresses a polypeptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide being capable of binding to human MDM2.

38. The method of claim 37 wherein said polypeptide comprises amino acids 1-41 of p53.

- 39. The method of claim 37 wherein said polypeptide comprises amino acids 13-57 of p53.
- 40. The method of claim 37 wherein said polypeptide comprises amino acids 1-50 of p53.
- 41. A polypeptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide capable of binding to human MDM2.
  - 42. The polypeptide of claim 41 which comprises amino acids 1-41 of p53.
  - 43. The polypeptide of claim 41 which comprises amino acids 13-57 of p53.
  - 44. The polypeptide of claim 41 which comprises amino acids 1-50 of p53.
- 45. The preparation of claim 21 wherein the antibodies do not bind to other human proteins.
- 46. The preparation of claim 21 wherein the antibodies do not bind to human proteins of M<sub>r</sub> 75-85K, 105-120K, and 170-200K.
- 47. The preparation of claim 21 wherein the antibodies bind to the epitope bound by antibodies secreted by hybridoma IF2 (ATCC HB 11290).
- 48. The preparation of claim 21 wherein the antibodies bind to the epitope bound by antibodies secreted by hybridoma ED9 (ATCC HB 11291).
- 49. The method of claim 7 wherein the antibodies bind to the epitope on hMDM2 bound by antibodies secreted by hybridoma IF2 (ATCC HB 11290).
- 50. The method of claim 4 wherein human MDM2 protein is detected by immunohistochemistry.
- 51. The method of claim 50 wherein antibodies are employed in the immunohistochemistry which bind to an epitope on hMDM2 bound by the antibodies secreted by ED9 (ATCC HB 11291).
- 52. The method of claim 50 wherein antibodies are employed in the immunohistochemistry which bind to an epitope on hMDM2 bound by the antibodies secreted by IF2 (ATCC HB 11290).

- 53. The method of claim 4 wherein human MDM2 protein is detected by immunoprecipitation.
- 54. A hybridoma cell having the identifying characteristics of ED9 (ATCC HB 11291).
- 55. A hybridoma cell having the identifying characteristics of IF2 (ATCC HB 11290).

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FIGURE

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FIGURE 1C

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p53 Ab1
p53 Ab2
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FIGURE 2

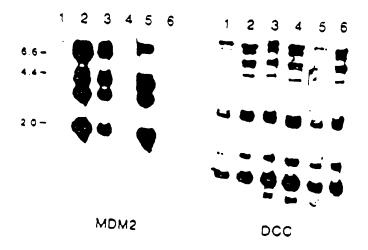


FIGURE 3

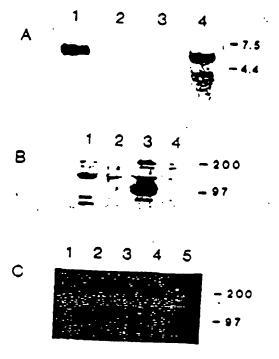


FIGURE 4

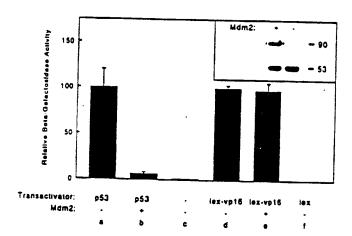


FIGURE 5

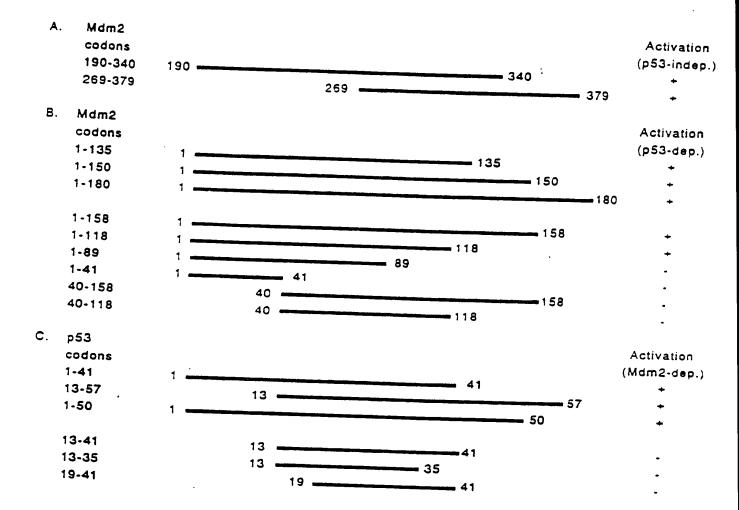


FIGURE 6

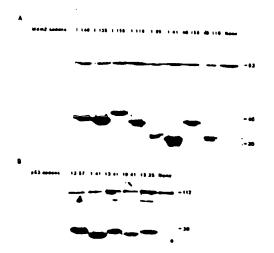


FIGURE 7

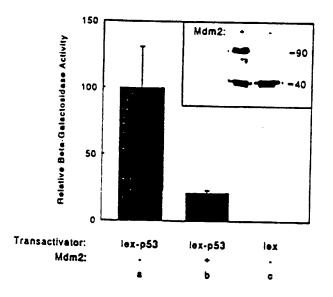


FIGURE 8

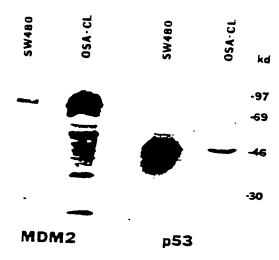


FIGURE 9

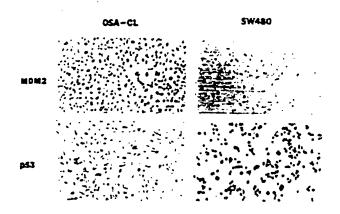


FIGURE 10



FIGURE 11









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PT, SE).

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(54) Title: AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

#### (57) Abstract

A human gene has been discovered which is genetically altered in human tumor cells. The genetic alteration is gene amplification and leads to a corresponding increase in gene products. Detecting that the gene, designated hMDM2, has become amplified or detecting increased expression of gene products is diagnostic of tumorigenesis. Human MDM2 protein binds to human p53 and allows the cell to escape from p53-regulated growth.

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# AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

This application is a continuation-in-part of United States Serial No. 07/903,103, filed June 23, 1992, which is a continuation-in-part of United States Serial No. 07/867,840, filed April 7, 1992, now abandoned.

This invention was made with support from the U.S. Government, including NIH grants CA-57345, CA-43460, CA-02243 and CA-35494. Accordingly, the Government retains certain rights in the invention.

### FIELD OF THE INVENTION

The invention relates to the area of cancer diagnostics and therapeutics. More particularly, the invention relates to the detection of a gene which is amplified in certain human tumors.

## BACKGROUND OF THE INVENTION

According to the Knudson model for tumorigenesis (Cancer Research, 1985, vol. 45, p. 1482), there are tumor suppressor genes in all normal cells which, when they become non-functional due to mutation, cause neoplastic development. Evidence for this model has been found in cases of retinoblastoma and colorectal tumors. The implicated suppressor genes in these tumors, RB and p53 respectively, were found to be deleted or altered in many of the tumors studied.

The p53 gene product, therefore, appears to be a member of a group of proteins which regulate normal cellular proliferation and suppression of cellular transformation. Mutations in the p53 gene have been linked to tumorigenesis, suggesting that alterations

p53 protein function are involved in cellular transformation. The inactivation of the p53 gene has been implicated in the genesis or progression of a wide variety of carcinomas (Nigro et al., 1989, Nature 342:705-708), including human colorectal carcinoma (Baker et al., 1989, Science 244:217-221), human lung cancer (Takahashi et al., 1989, Science 246:491-494; Iggo et al., 1990, Lancet 335:675-679), chronic myelogenous leukemia (Kelman et al., 1989, Proc. Natl. Acad. Sci. USA 86:6783-6787) and osteogenic sarcomas (Masuda et al., 1987, Proc. Natl. Acad. Sci. USA 84:7716-7719).

While there exists an enormous body of evidence linking p53 gene mutations to human tumorigenesis (Hollstein et al., 1991, Science 253:49-53) little is known about cellular regulators and mediators of p53 function.

Hinds et al. (Cell Growth & Differentiation, 1:571-580, 1990), found that p53 cDNA clones, containing a point mutation at amino acid residue 143, 175, 273 or 281, cooperated with the activated ras oncogene to transform primary rat embryo fibroblasts in culture. These mutant p53 genes are representative of the majority of mutations found in human cancer. Hollstein et al., 1991, Science 253:49-53. The transformed fibroblasts were found to produce elevated levels of human p53 protein having extended half-lives (1.5 to 7 hours) as compared to the normal (wild-type) p53 protein (20 to 30 minutes).

Mutant p53 proteins with mutations at residue 143 or 175 form an oligomeric protein complex with the cellular heat shock protein hsc70. While residue 273 or 281 mutants do not detectably bind hsc70, and are poorer at producing transformed foci than the 175 mutant, complex formation between mutant p53 and hsc70 is not required for p53-mediated transformation. Complex formation does, however, appear to facilitate this function. All cell lines transformed with the mutant p53 genes are tumorigenic in a thymic (nude) mice. In contrast, the wild-type human p53 gene does not possess transforming activity in cooperation with ras. Tuck and Crawford, 1989, Oncogene Res. 4:81-96.

Hinds et al., supra also expressed human p53 protein in transformed rat cells. When the expressed human p53 was immunoprecipitated with two p53 specific antibodies directed against distinct epitopes of p53, an unidentified  $M_r$  90,000 protein was coimmunoprecipitated. This suggested that the rat  $M_r$  90,000 protein is in a complex with the human p53 protein in the transformed rat cell line.

As mentioned above, levels of p53 protein are often higher in transformed cells than normal cells. This is due to mutations which increase its metabolic stability (Oven et al., 1981, Mol. Cell. Biol. 1:101-110; Reich et al. (1983), Mol. Cell. Biol. 3:2143-2150). The stabilization of p53 has been associated with complex formation between p53 and viral or cellular proteins. (Linzer and Levine, 1979, Cell 17:43-52; Crawford et al., 1981, Proc. Natl. Acad. Sci. USA 78:41-45; Dippold et al., 1981, Proc. Natl. Acad. Sci. USA 78:1695-1699; Lane and Crawford, 1979, Nature (Lond.) 278:261-263; Hinds et al., 1987. Mol. Cell. Biol. 7:2863-2869; Finlay et al., 1988, Mol. Cell. Biol. 8:531-539; Sarnow et al., 1982, Cell. 28:387-394; Gronostajski et al., 1984, Mol. Cell. Biol. 4:442-448; Pinhasi-Kimhi et al., 1986, Nature (Lond.) 320:182-185; Ruscetti and Scolnick, 1983, J. Virol. 46:1022-1026; Pinhasi and Oren, 1984, Mol. Cell. Biol. 4:2180-2186; and Sturzbecher et al., 1987, Oncogene 1:201-211.) For example, p53 protein has been observed to form oligomeric protein complexes with the SV40 large T antigen, the adenovirus type 5 E1B-M, 55,000 protein, and the human papilloma virus type 16 or 18 E6 product. Linzer and Levine, 1979. Cell 17:43-52; Lane and Crawford, 1979, Nature, 278:261-263; Sarnow et al., 1982, Cell 28:387-394; Werness et al., 1990, Science, 248:76-79. Similarly, complexes have been observed of p105RB (the product of the retinoblastoma susceptibility gene) with T antigen (DeCaprio et al., 1988, Cell 54:275-283), the adenovirus EIA protein (Whyte et al., 1988, Nature 334:124-129) and the E7 protein of human papilloma virus 16 or 18 (Münger et al., 1989, EMBO J. 8:4099-4105). It has been suggested that interactions between these viral proteins and p105RB inactivate a growth-suppressive function of p105<sup>RB</sup>, mimicking deletions and mutations commonly found in the RB gene in tumor cells. In a similar fashion, oligomeric protein complex

formation between these viral proteins and p53 may eliminate or alter the function of p53. Finlay et al., 1989, Cell 57:1083-1093.

Fakharzadeh et al. (EMBO J. 10:1565-1569, 1991) analyzed amplified DNA sequences present in a tumorigenic mouse cell line (i.e., 3T3DM, a spontaneously transformed derivative of mouse Balb/c cells). Studies were conducted to determine whether any of the amplified genes induced tumorigenicity following introduction of the amplified genes into a nontransformed recipient cell (e.g., mouse NIH3T3 or Rat2 cells). The resulting cell lines were tested for tumorigenicity in nude mice. A gene, designated MDM2, which is amplified more than 50-fold in 3T3DM cells, induced tumorigenicity when overexpressed in NIH3T3 and Rat 2 cells. From the nucleotide and predicted amino acid sequence of mouse MDM2 (mMDM2), Fakharzadeh speculated that this gene encodes a potential DNA binding protein that functions in the modulation of expression of other genes and, when present in excess, interferes with normal constraints on cell growth.

## SUMMARY OF THE INVENTION

It is an object of the invention to provide a method for diagnosing a neoplastic tissue, such as sarcoma, in a human.

It is another object of the invention to provide a cDNA molecule encoding the sequence of human MDM2.

Yet another object of the invention is to provide a preparation of human MDM2 protein which is substantially free of other human cellular proteins.

Still another object of the invention is to provide DNA probes capable of hybridizing with human MDM2 genes or mRNA molecules.

Another object of the invention is to provide antibodies immunoreactive with human MDM2 protein.

Still another object of the invention is to provide kits for detecting amplification or elevated expression of human MDM2.

Yet another object of the invention is to provide methods for identifying compounds which interfere with the binding of human MDM2 to human p53.

A further object of the invention is to provide a method of treating a neoplastic human cell.

Yet another object of the invention is to provide methods for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification.

Still another object of the invention is to provide polypeptides which interfere with the binding of human MDM2 to human p53.

A further object of the invention is to provide a method for growing host cells containing a p53 expression vector.

It has now been discovered that hMDM2, a heretofore unknown human gene, plays a role in human cancer. The hMDM2 gene has been cloned and the recombinant derived hMDM2 protein shown to bind to human p53 in vitro. hMDM2 has been found to be amplified in some neoplastic cells and the expression of hMDM2-encoded products has been found to be correspondingly elevated in tumors with amplification of this gene. The elevated levels of MDM2 appear to sequester p53 and allow the cell to escape from p53-regulated growth.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-C shows the cDNA sequence of human MDM2. In this figure, human and mouse nucleotide and amino acid sequences are compared, the mouse sequence being shown only where it differs from the corresponding human sequence.

Figure 2 shows that hMDM2 binds to p53.

Figure 3 illustrates the amplification of the hMDM2 gene in sarcomas.

Figure 4A-C illustrates hMDM2 expression.

Figure 5 shows the inhibition of p53-mediated transactivation by MDM2. Yeast were stably transfected with expression plasmids encoding p53, lex-VP16, MDM2 or the appropriate vector-only controls, as indicated. p53-responsive (bars a-c) or lexA-responsive (bars d-f)  $\beta$ -galactosidase reporter plasmids were used to assess the response.

Inset: Western blot analysis demonstrating MDM2 (90 kD) and p53 (53 kD) expression in representative yeast strains. The strain indicated by a plus was transfected with expression vector encoding full length MDM2 and p53, while the strain indicated by a minus was transfected only with the p53 expression vector.

Figure 6 shows the determination of MDM2 and p53 domains of interaction. Fig. 5A and Fig. 5B. Random fragments of MDM2 were fused to sequences encoding the lexA DNA binding domain and the resultant clones transfected into yeast carrying pRS314SN (p53 expression vector) and pJK103 (lexA-responsive  $\beta$ -galactosidase reporter). Yeast clones expressing  $\beta$ -galactosidase were identified by their blue color, and the MDM2 sequences in the lexA fusion vector were determined.  $\beta$ -galactosidase activity was observed independent of p53 expression in A, but was dependent on p53 expression in B. The bottom 6 clones in B were generated by genetic engineering. Fig. 6C. Random fragments of p53 were fused to the sequence encoding the B42 acidic activation domain and a hemagglutinin epitope tag; the resultant clones were transfected into yeast carrying lexA-MDM2 (lexA DNA binding domain fused to full length MDM2) and pJK103. Yeast clones were identified as above, and all were found to be MDM2-dependent. The bottom three clones were generated by genetic engineering.

Figure 7 shows protein expression from the yeast strains described in Figure 6. Western blot analysis was performed as described (Oliner, J.D., et al., Nature 358:80-83 (1992)), using 20 µg of protein per lane. The MDM2 and p53 codons contained in the fusion vectors are shown at the top of A and B, respectively. Fig. 7A. Upper panel probed with p53 Ab2 detecting p53; lower panel probed with anti-lexA polyclonal antibodies (lex Ab) detecting MDM2 fusion proteins of 30-50 kD. Fig. 7B. Upper panel probed with Lex Ab detecting the lexA-full length MDM2 fusion protein of 112 kD; lower panel probed with HA Ab (a monoclonal antibody directed against the hemagglutinin epitope tag, Berkeley Antibody) detecting p53 fusion proteins of approximately 25-30 kD.

- 7 -

Figure 8 shows the inhibition of the p53 activation domain by MDM2. Yeast were transfected with expression vectors encoding a lexA-p53 (p53 codons 1-73) fusion (bars a and b) or lexA alone (bar c). Strain b also expressed full length MDM2, and all strains contained the lexA-responsive  $\beta$ -galactosidase reporter plasmid. Inset: Upper panel probed with MDM2 polyclonal antibodies detecting full length MDM2 (90 kD); lower panel probed with lex Ab detecting the lex-p53 fusion protein of 40 kD.

Figure 9 shows a Western blot analysis using monoclonal antibodies to MDM2 or p53. Fifty  $\mu$ g of total cellular proteins from OsA-CL or SW480 cells were used for Western blot analysis. The position of molecular weight markers, in kd, is given on the right.

Figure 10 demonstrates immunocytochemical analysis of OsA-CL and SW480 cells grown in vitro. Monoclonal antibody IF-2, specific for MDM2, and mAb 1801, specific for p53, were used. The exclusively nuclear localization of both proteins is evident, as is the higher expression of MDM2 protein in OsA-CL cells than in SW480 cells, the reverse of the pattern observed for p53.

Figure 11 demonstrates MDM2 expression in primary soft tissue sarcomas. Cryostat sections of human sarcomas were incubated with the IF-2 antibody specific for MDM2. Tumors #3 and #10 showed nuclear expression of MDM2, while tumor #2 showed no staining.

## DETAILED DESCRIPTION OF THE INVENTION

It is a discovery of the present invention that a gene exists which is amplified in some human tumors. The amplification of this gene, designated MDM2, is diagnostic of neoplasia or the potential therefor. Detecting the elevated expression of human MDM2-encoded products is also diagnostic of neoplasia or the potential for neoplastic transformation. Over a third of the sarcomas surveyed, including the most common bone and soft tissue forms, were found to have amplified hMDM2 sequences. Expression of hMDM2 was found to be correspondingly elevated in tumors with the gene amplification.

Other genetic alterations leading to elevated hMDM2 expression may be involved in tumorigenesis also, such as mutations in regulatory regions of the gene. Elevated expression of hMDM2 may also be involved in tumors other than sarcomas including but not limited to those in which p53 inactivation has been implicated. These include colorectal carcinoma, lung cancer and chronic myelogenous leukemia.

According to one embodiment of the invention, a method of diagnosing a neoplastic tissue in a human is provided. Tissue or body fluid is isolated from a human, and the copy number of human MDM2 genes is determined. Alternatively, expression levels of human MDM2 gene products can be determined. These include protein and mRNA.

Body fluids which may be tested include urine, serum, blood, feces, saliva, and the like. Tissues suspected of being neoplastic are desirably separated from normal appearing tissue for analysis. This can be done by paraffin or cryostat sectioning or flow cytometry, as is known in the art. Failure to separate neoplastic from non-neoplastic cells can confound the analysis. Adjacent non-neoplastic tissue or any normal tissue can be used to determine a base-line level of expression or copy number, against which the amount of hMDM2 gene or gene products can be compared.

The human MDM2 gene is considered to be amplified if the cell contains more than the normal copy number (2) of this gene per genome. The various techniques for detecting gene amplification are well known in the art. Gene amplification can be determined, for example, by Southern blot analysis, as described in Example 4, wherein cellular DNA from a human tissue is digested, separated, and transferred to a filter where it is hybridized with a probe containing complementary nucleic acids. Alternatively, quantitative polymerase chain reaction (PCR) employing primers can be used to determine gene amplification. Appropriate primers will bind to sequences that bracket human MDM2 coding sequences. Other techniques for determining gene copy number as are known in the art can be used without limitation.

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The gene product which is measured may be either mRNA or protein. The term elevated expression means an increase in mRNA production or protein production over that which is normally produced by non-cancerous cells. Although amplification has been observed in human sarcomas, other genetic alterations leading to elevated expression of MDM2 may be present in these or other tumors. Other tumors include those of lung, breast, brain, colorectal, bladder, prostate, liver, skin, and stomach. These, too, are contemplated by the present invention. Non-cancerous cells for use in determining baseline expression levels can be obtained from cells surrounding a tumor, from other humans or from human cell lines. Any increase can have diagnostic value, but generally the mRNA or protein expression will be elevated at least about 3-fold, 5-fold, and in some cases up to about 100-fold over that found in non-cancerous cells. The particular technique employed for detecting mRNA or protein is not critical to the practice of the invention. Increased production of mRNA or protein may be detected, for example, using the techniques of Northern blot analysis or Western blot analysis, respectively, as described in Example 4 or other known techniques such as ELISA, immunoprecipitation, RIA and the like. These techniques are also well known to the skilled artisan.

According to another embodiment of the invention, nucleic acid probes or primers for the determining of human MDM2 gene amplification or elevated expression of mRNA are provided. The probe may comprise ribo- or deoxyribonucleic acids and may contain the entire human MDM2 coding sequence, a sequence complementary thereto, or fragments thereof. A probe may contain, for example, nucleotides 1-949, or 1-2372 as shown in Figure 1. Generally, probes or primers will contain at least about 14 contiguous nucleotides of the human sequence but may desirably contain about 40, 50 or 100 nucleotides. Probes are typically labelled with a fluorescent tag, a radioisotope, or the like to render them easily detectable. Preferably the probes will hybridize under stringent hybridization conditions. Under such conditions they will not hybridize to mouse MDM2. The probes of the invention are complementary to the human MDM2 gene. This means that they share 100% identity with the human sequence.

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hMDM2 protein can be produced, according to the invention, substantially free of other human proteins. Provided with the DNA sequence, those of skill in the art can express the cDNA in a non-human cell. Lysates of such cells provide proteins substantially free of other human proteins. The lysates can be further purified, for example, by immunoprecipitation, co-precipitation with p53, or by affinity chromatography.

The antibodies of the invention are specifically reactive with hMDM2 protein. Preferably, they do not cross-react with MDM2 from other species. They can be polyclonal or monoclonal, and can be raised against native hMDM2 or a hMDM2 fusion protein or synthetic peptide. The antibodies are specifically immunoreactive with hMDM2 epitopes which are not present on other human proteins. Some antibodies are reactive with epitopes unique to human MDM2 and not present on the mouse homolog. The antibodies are useful in conventional analyses, such as Western blot analysis, ELISA, immunohistochemistry, and other immunological assays for the detection of proteins. Techniques for raising and purifying polyclonal antibodies are well known in the art, as are techniques for preparing monoclonal antibodies. Antibody binding can be determined by methods known in the art, such as use of an enzyme-labelled secondary antibody, staphylococcal protein A, and the like. Certain monoclonal antibodies of the invention have been deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852. These include IF2, and ED9, which have been granted accession nos. HB 11290, and HB 11291, respectively.

According to another embodiment of the invention, interference with the expression of MDM2 provides a therapeutic modality. The method can be applied in vivo, in vitro, or ex vivo. For example, expression may be down-regulated by administering triple-strand forming or antisense oligonucleotides which bind to the hMDM2 gene or mRNA, respectively, and prevent transcription or translation. The oligonucleotides may interact with unprocessed pre-mRNA or processed mRNA. Small molecules and peptides which specifically inhibit MDM2 expression can also be used.

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Similarly, such molecules which inhibit the binding of MDM2 to p53 would be therapeutic by alleviating the sequestration of p53.

Such inhibitory molecules can be identified by screening for interference of the hMDM2/p53 interaction where one of the binding partners is bound to a solid support and the other partner is labeled. Antibodies specific for epitopes on hMDM2 or p53 which are involved in the binding interaction will interfere with such binding. Solid supports which may be used include any polymers which are known to bind proteins. The support may be in the form of a filter, column packing matrix, beads, and the like. Labeling of proteins can be accomplished according to any technique known in the art. Radiolabels, enzymatic labels, and fluorescent labels can be used advantageously. Alternatively, both hMDM2 and p53 may be in solution and bound molecules separated from unbound subsequently. Any separation technique known in the art may be employed, including immunoprecipitation or immunoaffinity separation with an antibody specific for the unlabeled binding partner.

It has been found that amino acid residues 13-41 of p53 (See SEQ ID NO:1) are necessary for the interaction of MDM-2 and p53. However, additional residues on either the amino or carboxy terminal side of the peptide appear also to be required. Nine to 13 additional p53 residues are sufficient to achieve MDM2 binding, although less may be necessary. Since cells which overexpress MDM2 escape from p53-regulated growth control in sarcomas, the use of p53-derived peptides to bind to excess MDM2 leads to reestablishment of p53-regulated growth control.

Suitable p53-derived peptides for administration are those which are circular, linear, or derivitized to achieve better penetration of membranes, for example. Other organic compounds which are modelled to achieve the same three dimensional structure as the peptide of the invention can also be used.

DNA encoding the MDM2-binding, p53-derived peptide, or multiple copies thereof, may also be administered to tumor cells as a mode of administering the peptide. The DNA will typically be in an expression construct, such as a retrovirus, DNA virus,

or plasmid vector, which has the DNA elements necessary for expression properly positioned to achieve expression of the MDM2-binding peptide. The DNA can be administered, *inter alia* encapsulated in liposomes, or in any other form known to the art to achieve efficient uptake by cells. As in the direct administration of peptide, the goal is to alleviate the sequestration of p53 by MDM2.

A cDNA molecule containing the coding sequence of hMDM2 can be used to produce probes and primers. In addition, it can be expressed in cultured cells, such as *E. coli*, to yield preparations of hMDM2 protein substantially free of other human proteins. The proteins produced can be purified, for example, with immunoaffinity techniques using the antibodies described above.

Kits are provided which contain the necessary reagents for determining gene copy number, such as probes or primers specific for the hMDM2 gene, as well as written instructions. The instructions can provide calibration curves to compare with the determined values. Kits are also provided to determine elevated expression of mRNA (i.e., containing probes) or hMDM2 protein (i.e., containing antibodies). Instructions will allow the tester to determine whether the expression levels are elevated. Reaction vessels and auxiliary reagents such as chromogens, buffers, enzymes, etc. may also be included in the kits.

The human MDM2 gene has now been identified and cloned. Recombinant derived hMDM2 has been shown to bind to human p53. Moreover, it has been found that hMDM2 is amplified in some sarcomas. The amplification leads to a corresponding increase in MDM2 gene products. Such amplification is associated with the process of tumorigenesis. This discovery allows specific assays to be performed to assess the neoplastic or potential neoplastic status of a particular tissue.

The following examples are provided to exemplify various aspects of the invention and are not intended to limit the scope of the invention.

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#### **EXAMPLES**

#### Example 1

To obtain human cDNA clones, a cDNA library was screened with a murine MDM2 (mMDM2) cDNA probe. A cDNA library was prepared by using polyadenylated RNA isolated from the human colonic carcinoma cell line CaCo-2 as a template for the production of random hexamer primed double stranded cDNA. Gubler and Hoffmann, 1983, Gene 25:263-268. The cDNA was ligated to adaptors and then to the lambda YES phage vector, packaged, and plated as described by Elledge et al. (Proc. Natl. Acad. Sci. USA, 88:1731-1735, 1991). The library was screened initially with a P-labelled (Kinzler, K.W., et al., Nucl. Acids Res. 17:3645-3653 (1989), Feinberg and Vogelstein, 1983, Anal. Biochem. 132.6-13) mMDM2 cDNA probe (nucleotides 259 to 1508 (Fakharzadeh et al., 1991, EMBO J. 10:1565-1569)) and then rescreened with an hMDM2 cDNA clone containing nucleotides 40 to 702.

Twelve clones were obtained, and one of the clones was used to obtain thirteen additional clones by re-screening the same library. In total, twenty-five clones were obtained, partially or totally sequenced, and mapped. Sequence analysis of the twenty-five clones revealed several cDNA forms indicative of alternative splicing. The sequence shown in Figure 1 is representative of the most abundant class and was assembled from three clones: c14-2 (nucleotides 1-949), c89 (nucleotides 467-1737), and c33 (nucleotides 390-2372). The 3' end of the untranslated region has not yet been cloned in mouse or human. The 5' end is likely to be at or near nucleotide 1. There was an open reading frame extending from the 5' end of the human cDNA sequence to nucleotide 1784. Although the signal for translation initiation could not be unambiguously defined, the ATG at nucleotide 312 was considered the most likely position for several reasons. First, the sequence similarity between hMDM2 and mMDM2 fell off dramatically upstream of nucleotide 312. This lack of conservation in an otherwise highly conserved protein suggested that the sequences upstream of the divergence may not code for protein. Second, an anchored polymerase chain reaction (PCR) approach was employed in an

effort to acquire additional upstream cDNA sequence. Ochman et al., 1985, In: PCR Technology: Principles and Applications for DNA Amplification (Erlich, ed.) pp. 105-111 (Stockton, New York). The 5' ends of the PCR derived clones were very similar (within 3 bp) to the 5' ends of clones obtained from the cDNA library, suggesting that the 5' end of the hMDM2 sequence shown in Figure 1 may represent the 5' end of the transcript. Third, in vitro translation of the sequence shown in Figure 1, beginning with the methionine encoded by the nucleotide 312 ATG, generated a protein similar in size to that observed in human cells.

In Figure 1, hMDM2 cDNA sequence, hMDM2 and mMDM2 nucleotide and amino acid sequences are compared. The mouse sequence is only shown where it differs from the corresponding human sequence. Asterisks mark the 5' and 3' boundaries of the previously published mMDM2 cDNA. Fakharzadeh et al., 1991, EMBO J. 10:1565-1569. Dashes indicate insertions. The mouse and human amino acid sequences are compared from the putative translation start site at nucleotide 312 through the conserved stop codon at nucleotide 1784.

Comparison of the human and mouse MDM2 coding regions revealed significant conservation at the nucleotide (80.3%) and amino acid (80.4%) levels. Although hMDM2 and mMDM2 bore little similarity to other genes recorded in current databases, the two proteins shared several motifs. These included a basic nuclear localization signal (Tanaka, 1990, FEBS Letters 271:41-46) at codons 181 to 185, several casein kinase II serine phosphorylation sites (Pinna, 1990, Biochem. et. Biophys. Acta. 1054:267-284) at codons 166 to 169, 192 to 195, 269 to 272, and 290 to 293, an acidic activation domain (Ptashne, 1988, Nature 355:683-689) at codons 223 to 274, and two metal binding sites (Harrison, 1991, Nature 353:715) at codons 305 to 322 and 461 to 478, neither of which is highly related to known DNA binding domains. The protein kinase A domain noted in mMDM2 (Fakharzadeh et al., 1991, EMBO J. 10:1565-1569) was not conserved in hMDM2.

#### Example 2

To determine whether the hMDM2 protein could bind to human p53 protein in vitro, an hMDM2 expression vector was constructed from the cDNA clones. The hMDM2 expression vector was constructed in pBluescript SK+ (Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in Figure 1 from nucleotide 312 to 2176. A 42 bp black bettle virus ribosome entry sequence (Dasmahapatra et al., 1987, Nucleic Acid Research 15:3933) was placed immediately upstream of this hMDM2 sequence in order to obtain a high level of expression. This construct, as well as p53 (El-Deriy et al., 1992, Nature Genetics, in press) and MCC (Kinzler et al., 1991, Science 251:1366-1370) constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Although the predicted size of the protein generated from the construct was only 55.2 kd (extending from the methionine at nucleotide 312 to nucleotide 1784), in vitro translated protein migrated at approximately 95 kilodaltons.

Ten  $\mu$ l of lysate containing the three proteins (hMDM2, p53 and MCC), alone or mixed in pairs, were incubated at 37°C for 15 minutes. One microgram (10  $\mu$ l) of p53 Ab1 (monoclonal antibody specific for the C-terminus of p53) or Ab2 (monoclonal antibody specific for the N-terminus of p53) (Oncogene Science), or 5  $\mu$ l of rabbit serum containing MDM2 Ab (polyclonal rabbit anti-hMDM2 antibodies) or preimmune rabbit serum (obtained from the rabbit which produced the hMDM2 Ab), were added as indicated. The polyclonal rabbit antibodies were raised against an *E. coli*-produced hMDM2-glutathione S-transferase fusion protein containing nucleotides 390 to 816 of the hMDM2 cDNA. Ninety  $\mu$ l of RIPA buffer (10 mM tris [pH 7.5], 1% sodium deoxycholate, 1% NP40, 150 mM NaCl, 0.1% SDS), SNNTE buffer, or Binding Buffer (El-Deriy et al., 1992, *Nature Genetics*, in press) were then added and the mixtures allowed to incubate at 4°C for 2 hours.

Two milligrams of protein A sepharose were added to each tube, and the tubes were rotated end-over-end at 4°C for 1 hour. After pelleting and washing, the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and the dried gels autoradiographed for 10 to 60 minutes in the presence of Enhance (New England Nuclear).

Figure 2 shows the co-precipitation of hMDM2 and p53. The three buffers produced similar results, although the co-precipitation was less efficient in SNNTE buffer containing 0.5 M NaCl (Figure 2, lanes 5 and 8) than in Binding Buffer containing 0.1 M NaCl (Figure 2 lanes 6 and 9).

In vitro translated hMDM2, p53 and MCC proteins were mixed as indicated above and incubated with p53 Ab1, p53 Ab2, hMDM2 Ab, or preimmune serum. Lanes 1, 4, 7, 10 and 14 contain aliquots of the protein mixtures used for immunoprecipitation. The bands running slightly faster than p53 are polypeptides produced from internal translation initiation sites.

The hMDM2 protein was not immunoprecipitated with monoclonal antibodies to either the C-terminal or N-terminal regions of p53 (Figure 2, lanes 2 and 3). However, when *in vitro* translated human p53 was mixed with the hMDM2 translation product, the anti-p53 antibodies precipitated hMDM2 protein along with p53, demonstrating an association *in vitro* (Figure 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility from another gene (MCC (Kinzler et al., 1991, *Science* 251:1366-1370)) was mixed with p53. No co-precipitation of the MCC protein was observed (Figure 2, lanes 8 and 9). When an *in vitro* translated mutant form of p53 (175hm) was mixed with hMDM2 protein, a similar co-precipitation of hMDM2 and p53 proteins was also observed.

In the converse of the experiments described above, the anti-hMDM2 antibodies immunoprecipitated p53 when mixed with hMDM2 protein (Figure 2, lane 15) but failed to precipitate p53 alone (Figure 5, lane 13). Preimmune rabbit serum failed to precipitate either hMDM2 or p53 (Figure 2, lane 16).

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#### Example 3

In order to ascertain the chromosomal localization of hMDM2, somatic cell hybrids were screened with an hMDM2 cDNA probe. A human-hamster hybrid containing only human chromosome 12 was found to hybridize to the probe. Screening of hybrids containing portions of chromosome 12 (Turc-Carel et al., 1986, Cancer Genet. Cytogenet. 23:291-299) with the same probe narrowed the localization to chromosome 12q12-14.

#### Example 4

Previous studies have shown that this region of chromosome 12 is often aberrant in human sarcomas. Mandahl et al., 1987, Genes Chromosomes & Cancer 1:9-14; Turc-Carel et al., 1986, Cancer Genet. Cytogenet. 23:291-299; Meltzer et al., 1991, Cell Growth & Differentiation 2:495-501. To evaluate the possibility that hMDM2 was genetically altered in such cancers, Southern blot analysis was performed.

Figure 3 shows examples of the amplification of the hMDM2 gene in sarcomas. Cellular DNA (5 μg) was digested with EcoRI, separated by agarose gel electrophoresis, and transferred to nylon as described by Reed and Mann (Nucl. Acids Res., 1985, 13:7207-7215). The cellular DNA was derived from five primary sarcomas (lanes 1-4, 6) and one sarcoma cell line (OsA-C1, lane 5). The filters were then hybridized with an hMDM2 cDNA fragment probe nucleotide 1-949 (see Figure 1), or to a control probe which identifies fragments of similar size (DCC gene, 1.65 cDNA fragment). Fearon, 1989, Science 247:49-56. Hybridization was performed as described by Vogelstein et al. (Cancer Research, 1987, 47:4806-4813). A striking amplification of hMDM2 sequences was observed in several of these tumors. (See Figure 3, lanes 2, 3 and 5). Of 47 sarcomas analyzed, 17 exhibited hMDM2 amplification ranging from 5 to 50 fold. These tumors included 7 to 13 liposarcomas, 7 of 22 malignant fibrous histiocytomas (MFH), 3 of 11 osteosarcomas, and 0 and 1 rhabdomyosarcomas. Five benign soft tissue tumors (lipomas) and twenty-seven carcinomas (colorectal or gastric) were also tested by Southern blot analysis and no amplification was observed.

#### Example 5

This example illustrates that gene amplification is associated with increased expression.

Figure 4A illustrates hMDM2 expression as demonstrated by Northern blot analysis. Because of RNA degradation in the primary sarcomas, only the cell lines could be productively analyzed by Northern blot. RNA was separated by electrophoresis in a MOPS-formaldehyde gel and electrophoretically transferred to nylon filters. Transfer and hybridization were performed as described by Kinzler et al. (Nature 332:371-374, 1988). The RNA was hybridized to the hMDM2 fragment described in Figure 3. Ten  $\mu$ g of total RNA derived, respectively, from two sarcoma cell lines (OsA-CL, lane 1 and RC13, lane 2) and the colorectal cancer cell line (CaCo-2) used to make the cDNA library (lane 3). Lane 4 contains 10  $\mu$ g of polyadenylated CaCo-2 RNA. RNA sizes are shown in kb. In the one available sarcoma cell line with hMDM2 amplification, a single transcript of approximately 5.5 kb was observed (Figure 4A, lane 1). The amount of this transcript was much higher than in a sarcoma cell line without amplification (Figure 4A, lane 2) or in a carcinoma cell line (Figure 4A, lane 3). When purified mRNA (rather than total RNA) from the carcinoma cell line was used for analysis, an hMDM2 transcript of 5.5 kb could also be observed (Figure 4A, lane 4).

Figure 4B illustrates hMDM2 expression as demonstrated by Western blot analysis of the sarcoma cell lines RC13 (lane 1), OsA-CL (lane 3), HOS (lane 4), and the carcinoma cell line CaCo-2 (lane 2).

Figure 4C illustrates hMDM2 expression as demonstrated by Western blot analysis of primary sarcomas. Lanes 1 to 3 contain protein from sarcomas with hMDM2 amplifications, and lanes 4 and 5 contain protein from sarcomas without hMDM2 amplification.

Western blots using affinity purified MDM2 Ab were performed with 50  $\mu$ g protein per lane as described by Kinzler et al. (Mol. Cell. Biol., 1990, 10:634-642), except that the membranes were blocked in 10% nonfat dried milk and 10% goat serum,

and secondary antibodies were coupled to horseradish peroxidase, permitting chemiluminescent detection (Amersham ECL). MDM2 Ab was affinity purified with a pATH-hMDM2 fusion protein using methods described in Kinzler et al. (*Mol. Cell. Biol. 10*:634-642, 1990). Non-specifically reactive proteins of about 75-85, 105-120 and 170-200 kd were observed in all lanes, irrespective of hMDM2 amplification status. hMDM2 proteins, of about 90-97 kd, were observed only in the hMDM2-amplified tumors. Protein marker sizes are shown in kd.

A protein of approximately 97 kilodaltons was expressed at high levels in the sarcoma cell line with hMDM2 amplification (Figure 4B, lane 3), whereas no expression was evident in two sarcoma cell lines without amplification or in the carcinoma cell line (Figure 4B, lanes 1, 2 and 4). Five primary sarcomas were also examined by Western blot analysis. Three primary sarcomas with amplification expressed the same size protein as that observed in the sarcoma cell line (Figure 4C, lanes 1-3), while no protein was observed in the two sarcomas without amplification (Figure 4C, lanes 4 and 5).

Expression of the hMDM2 RNA in the sarcoma with amplification was estimated to be at least 30 fold higher than that in the other lines examined. This was consistent with the results of Western blot analysis.

The above examples demonstrate that hMDM2 binds to p53 in vitro and is genetically altered (i.e., amplified) in a significant fraction of sarcomas, including MFH, liposarcomas, and osteosarcomas. These are the most common sarcomas of soft tissue and bone. Weiss and Enzinger, 1978, Cancer 41:2250-2266; Malawer et al., 1985, In: Cancer: Principles and Practice of Oncology, DeVita et al., Eds., pp. 1293-1342 (Lippincott, Philadelphia).

Human MDM2 amplification is useful for understanding the pathogenesis of these often lethal cancers.

MDM2 may functionally inactivate p53 in ways similar to those employed by virally encoded oncoproteins such as SV40 T-antigen, adenovirus E1B, and HPV E6. Lane and Bechimol. 1990. Genes and Development 4:1-8; Werness et al., 1990, Science

248:76. Consistent with this hypothesis, no sarcomas with hMDM2 amplification had any of the p53 gene mutations that occur commonly in other tumors. hMDM2 amplification provides a parallel between viral carcinogenesis and the naturally occurring genetic alterations underlying sporadic human cancer. The finding that expression of hMDM2 is correspondingly elevated in tumors with amplification of the gene are consistent with the finding that MDM2 binds to p53, and with the hypothesis that overexpression of MDM2 in sarcomas allows escape from p53 regulated growth control. This mechanism of tumorigenesis has striking parallels to that previously observed for virally induced tumors (Lane and Bechimol, 1990, Genes and Development 4:1-8; Werness et al., 1990, Science 248:76), in which viral oncogene products bind to and functionally inactivate p53.

#### Example 6

This example demonstrates that MDM2 expression inhibits p53-mediated transactivation.

To determine if MDM2 could influence the ability of p53 to activate transcription, expression vectors coding for the two proteins were stably transfected into yeast along with a p53-responsive reporter construct. The reporter consisted of a β-galactosidase gene under the transcriptional control of a minimal promoter and a multimerized human DNA sequence which strongly bound p53 in vitro (Kern, S.E., et al., Science 256:827-830 (1992). Reporter expression was completely dependent on p53 in this assay (Figure 5, compare bars a and c). MDM2 expression was found to inhibit p53-mediated transactivation of this reporter 16-fold relative to isogeneic yeast lacking MDM2 expression (Figure 5, compare bars a and b). Western blot analysis confirmed that p53 (53 kD) was expressed equivalently in strains with and without MDM2 (90 kD) (Figure 1, inset).

METHODS. The MDM2 expression plasmid, pPGK-MDM2, was constructed by inserting the full length MDM2 cDNA (Oliner, J.D., et al., Nature 358:80-83 (1992)) into pPGK (Poon, D. et al., Mol. and Cell.

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Biol. 1111:4809-4821 (1991)), immediately downstream of the phosphoglycerate kinase constitutive promoter. Galactose-inducible p53 (pRS314SN, Nigro, J.M., et al., Mol. and Cell. Biol. 12:1357-1365 (1992)), lexA-VP16 (YVLexA, Dalton, S., et al., Cell 68:597-612 (1992)), and lexA (YLexA, YVLexA minus VP16) plasmids were used as indicated. The reporters were PG16-lacZ (Kern, S.E. et al., Science 256:827-830 (1992)) (p53-responsive) and pJK103 (Kamens, J., et al., Mol. Cell. Biol. 10:2840-2847 (1990)) (lexA-responsive). S. cerevisiae strain pEGY48 was transformed as described (Kinzler, K.W. et al., Nucl. Acids Res. 17:3645-3653 (1989)). Yeast strains represented by bars a-c were grown at 30°C to mid-log phase in selective liquid medium containing 2% raffinose as the carbon source, induced for 30 minutes by the addition of 2% galactose, harvested, and lysed as described (Kern, S.E. et al., Science 256:827-830 (1992)). The strains represented by bars d-f were treated similarly, except that the cells were induced in galactose for 4 hours to obtain measurable levels of  $\beta$ -galactosidase.  $\beta$ -galactosidase activities shown represent the mean of three to five experimental values (error bars indicate s.e.m.). Protein concentrations were determined by à Coomassie blue-based (bio-Rad) assay. The  $\beta$ -galactosidase assays were performed with AMPGD chemiluminescent substrate and Emerald enhancer (Tropix) according to the manufacturer's instructions. galactosidase activities of bars b and c are shown relative to that of bar A;  $\hat{\beta}$ -galactosidase activities of bars e and f are shown relative to that of bar Western blots were performed as described (Oliner, J.D., et al., Nature 358:80-83 (1992)), using p53 Ab1801 (lower panel, Oncogene Science) or MDM2 polyclonal antibodies (Oliner, J.D., et al., Nature 358:80-83 (1992)) (upper panel).

To ensure that this inhibition was not simply a general transcriptional down regulation mediated by the expression of the foreign MDM2 gene, a yeast strain was created that contained a different transcriptional activator (lexA-VP16, consisting of the lexA DNA binding domain fused to the VP16 acidic activation domain), a similar reporter (with a lexA-responsive site upstream of a  $\beta$ -galactosidase gene), and the same MDM2 expression vector. The results shown in Figure 1 (bars d & e) demonstrate that lexA-VP16 transactivation was unaffected by the presence of MDM2. Furthermore, MDM2 expression had no apparent effect on the growth rate of the cells.

#### Example 7

This example demonstrates the domains of p53 and MDM2 which interact with each other.

To gain insight into the mechanism of the MDM2-mediated p53 inhibition, the domains of MDM2 and p53 responsible for binding to one another were mapped. The yeast system used to detect protein-protein binding takes advantage of the modular nature of transcription factor domains (Keegan, L., et al., Science 231:699-704 (1986); Chien, C.-T., Proc. Natl. Acad. Sci. U.S.A. 88:9578-9582 (1991); Brent, R., et al., Cell 43:729-731 (1985); Ma, J., et al., Cell 55:4430446 (1988). Generically, if protein 1 (fused to a sequence-specific DNA binding domain) is capable of binding to protein 2 (fused to a transcriptional activation domain), then co-expression of both fusion proteins will result in transcriptional activation of a suitable reporter. In our experiments, the lexA DNA binding domain (amino acids 2-202) and the B42 acidic activation domain (AAD) were used in the fusion constructs. The reporter (Kamens, J., et al., Mol. Cell. Biol. 10:2840-2847 (1990); contained a lexA-responsive site upstream of a  $\beta$ galactosidase gene. As an initial control experiment, full length MDM2 was inserted into the lexA fusion vector, and full length p53, supplying its intrinsic activation domain was inserted into a non-fusion vector. The combination resulted in the activation of the lexAresponsive reporter, while the same expression constructs lacking either the MDM2 or p53 cDNA inserts failed to activate  $\beta$ -galactosidase (Table I, strains 1, 2, and 3). Thus, activation was dependent upon MDM2-p53 binding.

This assay was then applied to mapping the interaction domains of each protein. Full length cDNA fragments encoding MDM2 or p53 were randomly sheared by sonication, amplified by polymerase chain reaction, size fractionated, cloned into the appropriate fusion vectors and transfected into yeast along with the reporter and the full length version of the other protein.

METHODS. Full length MDM2 cDNA in pBluescript SK+(Stratagene) was digested with XhoI and BamHI to excise the entire insert. After agarose gel purification, the insert was sheared into random fragments by sonication, polished with the Klenow fragment of DNA polymerase I, ligated to catch linkers, and amplified by the polymerase chain reaction as described (Kinzler, K.W., et al., Nucl. Acids Res. 17:3645-3653 (1989)). The fragments were fractionated on an acrylamide gel into size ranges of 100-400 bp or 400-1000 pb, cloned into lexA(1-202)+PL (Ruden, D.M., et al., Nature 350:250-252 (1991)), and transfected into bacteria (XL-1 Blue, Stratagene). At least 10,000 bacterial colonies were scraped off agar plates, and the plasmid DNA was transfected into a strain of pEGY48 containing pRS314N (p53 expression vector) and pJK103 (lexA-responsive  $\beta$ -galactosidase reporter). Approximately 5,000 yeast clones were plated on selective medium containing 2% dextrose, and were replica-plated onto glalctose- and X-gal-containing selective medium. Blue colonies (17) appeared only on the plates containing the larger fragments of MDM2. The 17 isolated colonies were tested for blue color in this assay both in the presence and in the absence of galactose (p53 induction); all tested positive in the presence of galactose but only 2 of the 17 tested positive in its absence. MDM2-containing plasmid DNA extracted from the 17 yeast clones was selectively transferred to bacterial strain KC8 and sequenced from the lexA-MDM2 junction. The MDM2 sequences of the two p53independent clones are diagrammed in Fig. 6A. The MDM2 sequences of the remaining 15 p53-dependent clones coded for peptides ranging from 135 to 265 a.a. in length and began exclusively at the initiator methionine. Three of the MDM2 sequences obtained are shown at the top of Fig. 6B. The lower 6 sequences were genetically engineered (using the polymerase chain reaction and appropriate primers) into lexA(1-202)+PL and subsequently tested to further narrow the binding region.

Fragments of p53 were also cloned into pJG4-5, producing a fusion protein C-terminal to the B42 acidic activation domain and incorporating an epitope of hemagglutinin. The clones were transfected into a strain of pEGY48 already containing lex-MDM2 (plex-202+PL containing full length MDM2) and pJK103. The top three p53 sequences shown in Fig. 6C. were derived from yeast obtained by colony screening, whereas the lower three were genetically engineered to contain the indicated fragments.

The resultant yeast colonies were examined for  $\beta$ -galactosidase activity in situ. Of approximately 5000 clones containing MDM2 fragments fused to the lexA DNA

binding domain, 17 were found to score positively in this assay. The clones could be placed into two classes. The first class (two clones) expressed low levels of  $\beta$ galactosidase (about 5-fold less than the other fifteen clones) and  $\beta$ -galactosidase expression was independent of p53 expression (Figure 6A). These two clones encoded MDM2 amino acids 190-340 and 269-379, respectively. The region shared between these two clones overlapped the only acidic domain in MDM2 (amino acids 230-301). This domain consisted of 37.5% aspartic and glutamic acid residues but no basic amino acids. This acidic domain appears to activate transcription only when isolated from the rest of the MDM2 sequence, because the entire MDM2 protein fused to lexA had no measurable  $\beta$ -galactosidase activity in the same assay (Table I, strain 3). The other class (15 clones) each contained the amino terminal region of MDM2 (Figure 6B). The  $\beta$ -galactosidase activity of these clones was dependent on p53 co-expression. To narrow down the region of interaction, we generated six additional clones by genetic engineering. The smallest tested region of MDM2 which could functionally interact with full length p53 contained MDM2 codons 1 to 118 (Figure 6B). The relatively large size of the domain required for interaction was consistent with the fact that when small sonicated fragments of MDM2 were used in the screening assay (200 bp instead of 600 bp average size), no positively scoring clones were obtained.

In a converse set of experiments, yeast clones containing fragments of p53 fused to the B42 AAD were screened for lexA-responsive reporter expression in the presence of a lexA-MDM2 fusion protein. Sequencing of the 14 clones obtained in the screen revealed that they could be divided into three subsets, one containing amino acids 1-41, a second containing amino acids 13-57, and a third containing amino acids 1-50 (Figure 2C). The minimal overlap between these three fragments contained codons 13-41. Although this minimal domain was apparently necessary for interaction with MDM2, it was insufficient, as the fragments required 9-12 amino acids on either side of codons 13-41 for activity (Figure 6C). To further test the idea that the amino terminal region of p53 was required for MDM2 binding, we generated an additional yeast strain expressing

the lexA-DNA binding domain fused to p53 codons 74-393) and the B42 acidic activation domain fused to full length MDM2. These strains failed to activate the same lexA-responsive reporter (Table I, strain 8), as expected if the N-terminus of p53 were required for the interaction.

TABLE I

STRAIN NUMBER	p53 CONSTRUCT	MDM2 CONSTRUCT	ACTIVATION
1	p53*	Vector	-
2	p53*	lexA-MDM2b	+
3	Vector*	lexA-MDM2b	-
4	p53°	lexA-MDM2 (1-118) <sup>b</sup>	+
5	Vector*	lexA-MDM2 (1-118) <sup>b</sup>	-
6	B42-p53 (1-41)°	lexA-MDM2	+
7	B42-p53 (1-41)°	Vector*	-
8	lexA-p53 (74-393) <sup>b</sup>	B42-MDM2°	-
9	p53 (1-137)*	lexA-MDM2b	-

The MDM2 and p53 proteins expressed in each strain, along with the relevant reporters, are indicated. Numbers in parentheses refer to the MDM2 or p53 amino acids encoded (absence of parentheses indicated full length protein, that is, MDM2 amino acids 1 to 491 or p53 amino acids 1 to 393). The lexa-responsive  $\beta$ -galactosidase reporter plasmid (pJK103, Kamens, J., et al., Mol. Cell. Biol. 10:2840-2847 (1990)) was present in all strains.

pRS314 vector (Nigro, J.M., et al., Mol. and Cell. Biol. 12:1357-1365 (1992).

plex(1-202)+PL vector, containing lexA DNA binding domain fused to insert (Ruden, D.M., et al., Nature 350:250-252 (1991).

pJG4-5 vector, containing B42 activation domain fused to insert.

 $^4(+)$  indicates that colonies turned blue following 24 hours of incubation on X-gal-containing selective medium, while (-) indicates that colonies remained white following 72 hours of incubation.

Sequence analysis showed that all p53 and MDM2 fragments noted in Figure 6 were ligated in frame and in the correct orientation relative to the B42 and lexA domains, respectively. Additionally, all clones compared in Figure 6 expressed the relevant proteins at similar levels, as shown by Western blotting (Figure 7).

The most striking results of these mapping experiments was that the region of p53 required to bind MDM2 was almost identical to the previously identified acidic activation domain of p53 (amino acids 20-42) (Unger, T., et al., EMBO J. 11:1383-1390 (1992); Miller, C.W., et al., Proc. Am. Assoc. Cancer Res. 33:386 (1992). This suggested that MDM2 inhibits p53-mediated transcriptional activation by "concealing" the activation domain of p53 from the transcriptional machinery. If this were true, the p53 activation domain, in isolation from the rest of the p53 protein, should still be inhibitable by full length MDM2. To test this hypothesis, we produced a hybrid protein containing the p53 activation domain (codons 1-73) fused to the lexA-DNA binding domain. This construct exhibited strong transcriptional activation of a lexA-responsive reporter (Figure 8), as predicted from previous experiments in which the p53 activation domain was fused to another DNA binding domain (Fields, S., et al., Science 249:1046-1049 (1990); Raycroft, L., et al., Science 249:1049-1051 (1990)). The lexA-p53 DNA construct was stably expressed in yeast along with the full length MDM2 expression vector (or the vector alone). MDM2 expression resulted in a five-fold decrease in reporter activity, demonstrating that MDM2 can specifically inhibit the function of the p53 activation domain regardless of the adjacent protein sequences tethering p53 to DNA (Figure 8).

METHODS. Strains were grown to mid-log phase in 2% dextrose before induction of p53 expression for 2 hours by the addition of 2% galactose. The lex-p53 construct was identical to lex-VP16 (YVlexA, Dalton, S., et al., Cell 68:597-612 (1992)) except that VP16 sequences were replaced by p53 sequences encoding amino acids 1 to 73.

The results obtained in the experiments discussed herein raise an interesting paradox. If MDM2 binds to (Figure 6) and conceals (Figure 8) the p53 activation

domain from the transcriptional machinery, how could the lexA-MDM2-p53 complex activate transcription from the lexA-responsive reporter (Table I, strain 2)? Because the only functional activation domain in the lexA-MDM2-p53 complex of strain 2 is expected to be contributed by p53, one might predict that it would be concealed by binding to MDM2 and thereby fail to activate. A potential resolution of this paradox is afforded by knowledge that p53 exists as a homotetramer (Stenger, J.E., et al., Mol. Carcinogenesis 5:102-106 (1992); Sturzbecher, H.W. et al., Oncogene 7:1513-1523 (1992). Thus the activation noted in the lexA-MDM2-p53 complex could be due to the presence of four individual activation domains contributed by the p53 tetramer, not all of which were concealed by MDM2. As a direct test of this issue, the domain of p53 required for homo-oligomerization (Stenger, J.E., et al., Mol. Carcinogenesis 5:102-106 (1992); Sturzbecher, H.W. et al., Oncogene 7:1513-1523 (1992) (the C-terminus) was removed from the p53 expression construct, so that it consisted of only codons 1-137. This truncated p53 polypeptide retained the entire activation domain (as shown in Figure 8, bar a) and the entire domain required for interaction with MDM2 (Table I, strain 6). Yet, when allowed to interact with lexA-MDM2, no transactivation of the lexAresponsive reporter was observed (Table I, strain 9). Because p53 did not inhibit lexA-MDM2 binding to the lexA reporter (Table I, strain 2), the result of strain 9 is likely to be due to a direct inhibition of the isolated p53 activation domain by MDM2.

#### Example 8

This example illustrates the production and characterization of antibodies specific for MDM2 epitopes.

The antigen preparations used to intraperitoneally immunize female (BALB/c X C57BL/5)F1 mice comprised bacterially expressed, glutathione-column purified glutathione-S-transferase-MDM2 (GST-MDM2) fusion protein. (One preparation was further purified on a polyacrylamide gel and electroeluted.) The fusion protein contains a 16 kD amino terminal portion of human MDM2 protein (amino acids 27 to

168). For immunization, the fusion protein was mixed with Ribi adjuvant (Ribi Immunochem Research, Inc.).

Two mice were sacrificed and their spleen cells fused to SP2/0s myeloma cells (McKenzie, et al., Oncogene, 4:543-548, 1989). Resulting hybridomas were screened by ELISA on trpE-MDM2 fusion protein-coated microtiter wells. The trpE-MDM2 fusion protein contains the same portion of MDM2 as the GST-MDM2 fusion protein. Antigen was coated at a concentration of  $1 \mu g/ml$ .

A second fusion was performed as described except hybridomas were screened on electroeluted, glutathione purified GST-MDM2. Positive hybridomas from both fusions were expanded and single cell subcloned. Subclones were tested by Western Blot for specificity to the 55 kD trpE-MDM2 and the 43 kD GST-MDM2 fusion proteins.

Two Western Blot positive subclones (1F2 and JG3) were put into mice for ascites generation. The resulting ascites were protein A purified. Both purified monoclonal antibodies tested positive by Western Blot and immunoprecipitation for the 90 kD migrating MDM2 protein present in a human osteosarcoma cell line (OsA-CL), which overexpresses MDM2, and negative in the HOS osteosarcoma, which does not overexpress MDM2.

ED9 was protein G-purified from ascites and found to be specific in cryostat immunohistochemistry for MDM2 in osteosarcoma cells, as was IF2.

### Example 9

This example demonstrates the expression and detection of MDM2 at the cellular level.

To evaluate MDM2 expression at the cellular level, we produced monoclonal antibodies against bacterially generated fusion proteins containing residues 27 to 168 of MDM2. (See example 8.) Of several antibodies tested, mAb IF-2 was the most useful, as it detected MDM2 in several assays. For initial testing, we compared proteins derived

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from OsA-CL, a sarcoma cell line with MDM2 amplification but without p53 mutation (Table II) and proteins from SW480, a colorectal cancer cell line with p53 mutation (Barak et al., EMBO 12:461-468 (1993)) but without MDM2 amplification (data not shown). Figure 9 shows that the mAb IF-2 detected an intense 90 kd band plus several other bands of lower molecular weight in OsA-CL extracts, and a much less intense 90 kd band in SW480 extracts. We could not distinguish whether the low molecular weight bands in OsA-CL were due to protein degradation or alternative processing of MDM2 transcripts. The more than 20-fold difference in intensity between the signals observed in OsA-CL and SW480 is consistent with the greater than 20-fold difference in MDM2 gene copy number in these two lines. Conversely, the 53 kd signal detected with p53-specific mAb 1801 was much stronger in SW480 than in OsA-CL consistent with the presence of a mutated p53 in SW480 (Fig. 9).

Cells grown on cover slips were then used to assess the cellular localization of the MDM2 protein. A strong signal, exclusively nuclear, was observed in OsA-CL cells with the IF-2 mAb and a weaker signal, again strictly nuclear, was observed in SW480 (Fig. 10). The nuclear localization of MDM2 is consistent with previous studies of mouse cells (Barak et al., EMBO 12:461-468 (1993)) and the fact that human MDM2 contains a nuclear localization signal at residues 179 to 186. Reactivity with the p53-specific antibody was also confined to the nuclei of these two cell lines (Fig. 10), with the relative intensities consistent with the Western blot results (Fig. 9).

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The IF-2 mAb was then used (at 5  $\mu$ g/ml) to stain the seven primary sarcomas noted above. The nuclei of two of them (tumors #3 and #10) stained strongly (Fig. 11). Both of these tumors contained MDM2 gene amplification (Table II). In the five tumors without amplification, little or no MDM2 reactivity was observed (example in Fig. 11).

# TABLE II

TUMOR	TUMOR	TYPE	MDM2	P53	OVER-
	M.	102	NOT TOUT TO	ALTERATION	EXPRESSION
	7 H	H1E	ABSENT	DELETION/ REARRANGEMENT	NONE
2	M-5	MFH	ABSENT	CGC-CUC MUTATION;	p53
3	M-7	MFH		SIH- (BCI)614	
-			PRESENT.	NONE OBSERVED	MDM2
	8-6	HEH	ABSENT	DELETION	NONE
6	M-14	MFH	ABSENT	NONE ORSEDVED	
9	M-15	MFH	ABGENT	COSTUNED	N. T.
7	M-16			DELETION	N.T.
	$\dagger$	31.0	ABSENT	NONE OBSERVED	NONE
8	M-17	MFII	ABSENT		
σ				NONE OBSERVED	N.T.
Ì	01_6	HTH	ABSENT	OVEREXPRESSED	n£1
10	M-20	MFH	PRESENT	NONE Opening	CCA
11	L-5	LIPOSABCOMA		TONE OBSERVED	MDM2
:	$\dagger$	╁	ABSENT.	NONE OBSERVED	N.T.
71	Γ-1	LIPOSARCOMA	ABSENT	AAC-AGC MUTATION;	N.T.
13	L-9 I	LIPOSARCOMA	PRESENT	Jac- (667)	
		-	-	CONF. ORCEDUDO	

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# TABLE II (Cont.)

TUMOR	TUMOR	TYPE	MDM2 AMPLIFICATION <sup>D</sup>	P53	OVER
14	[-1]	LIDOCABCOMA		NOTIVE	EXPRESSION <sup>o</sup>
		THE COUNTY	ADSENT	NONE OBSERVED	Z.T.
15	KL5B	LIPOSARCOMA	ABSENT	CAG-UAG MUTATION;	N.T.
16	KL7	LIPOSARCOMA	DDFCFNT	done (thi)	
17	V 17		THE CONTRACT	NONE OBSERVED	N.T.
	O TOTAL	LIFUSARCOMA	ABSENT	NONE OBSERVED	N.T.
18	KL11	LIPOSARCOMA	ABSENT	GGT-GAT MUTATION; EXON 5	N.T.
19	KL12	LIDOSABCOMA	min 00 4	STORY STIE	
		AEOONACO TA	ABSENT	NONE OBSERVED	N.T.
20	KI.28	LIPOSARCOMA	PRESENT	NONE OBSERVED	£ 2
21	KL30	LIPOSARCOMA	PRESENT	NONE OBSERVED	
22	S189	LIPOSARCOMA	PRESENT	day and anon	N. I.
. 23	SIJIB	LIBOCABOOM		NONE OBSERVED	N.T.
		3	ADSENT	NONE OBSERVED	N.T.
24	OSA-CL	MFH	PRESENT	NONE OBSERVED	MOM
					217013

MFH= malignant fibrous histiocytoma

b as assessed by Southern blot

<sup>c</sup> as assessed by Southern blot, sequencing of exons 5-8, or immunohistoch**em**ical analysis

 $^{
m d}$  as assessed by immunohistochemical analysis; N.T.  $^{\pm}$  not tested

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#### SEQUENCE LISTING

#### (1) GENERAL INFORMATION:

- (i) APPLICANT: BURRELL, MARILEE
  HILL, DAVID E.
  KINZLER, KENNETH W.
  VOGELSTEIN, BERT
- (ii) TITLE OF INVENTION: AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS
- (iii) NUMBER OF SEQUENCES: 5
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  - (E) COUNTRY: USA
  - (F) ZIP: 20001
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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#### (vill) ATTORNEY/AGENT INFORMATION:

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- (B) REGISTRATION NUMBER: 32,141
- (C) REFERENCE/DOCKET NUMBER: 01107.42798

#### (ix) TELECOMMUNICATION INFORMATION:

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- (B) TELEFAX: 202-508-9299
- (C) TELEX: 197430 BBMB UT

#### (2) INFORMATION FOR SEQ ID NO:1:

- (1) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 64 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

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- (v) FRAGMENT TYPE: N-terminal
- (V1) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
- (viii) POSITION IN GENOME:
  - (A) CHROMOSOME/SEGMENT: 17q
  - (x1) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln

1 10 15

Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu Asn Asn Val Leu 20 25 30

Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Leu Ser Pro Asp 35 40 45

Asp Ile Glu Gln Trp Phe Thr Glu Asp Pro Gly Pro Asp Glu Ala Pro 50 55 60

- (2) INFORMATION FOR SEQ ID NO:2:
  - (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2372 base pairs

(B) TYPE: nucleic acid

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(C) STRANDEDNESS: double	
(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: cDNA	
	•
(iii) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: NO	
(vi) ORIGINAL SOURCE:	
(A) ORGANISM: Homo sapiens	
(H) CELL LINE: CaCo-2	
(iii)	
(viii) POSITION IN GENOME:	
(B) MAP POSITION: 12q12-14	
(ix) FEATURE:	
(A) NAME/KEY: CDS	
(B) LOCATION: 3121784	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:	
GCACCGCGCG AGCTTGGCTG CTTCTGGGGC CTGTGTGTCG GAAAGATGGA	60
GCANGANGCC CACCOURAGE CONTRACT	
GCAAGAAGCC GAGCCCGAGGCCGGGGCGGCG ACCCCTCTGA CCGAGATCCT GCTGCTTTCG	120
CAGCCAGGAG CACCGTCCCT CCCCGGATTA GTGCGTACGA GCGCCCAGTG CCCTGGCCCG	
	180

GAG	AGTG	GAA	TGAT	rccc	CGA G	GCCC	AGGG	C GI	CGTG	CTT	CGC	AGTA	GTC	AGTO	ccc	GTG	240
AAG	GAAA	CTG	GGGZ	GTCT	TG A	GGGA	CCCC	C GA	.CTCC	AAGC	GCG	AAAA	ccc	CGGZ	TGGT	.GA	300
GGA	GCAG	GCA										T AC					350
				1				5					.0	p G.	Ly A.	ıa	
												ACC					398
vai	15	Thr	Ser	Gln	Ile	Pro 20		Ser	Glu	Glr	Glu 25	Thr	Leu	ı Val	. Arg	a .	
CCA	AAG	CCA	TTG	CTT	TTG	AAG	TTA	TTA	AAG	TCT	GTT	GGT	GCA	CAA	AAA		446
												Gly					
30					35					40					4 5		
												GGC					494
Asp	Thr	Tyr	Thr		Lys	Glu	Val	Leu	Phe	Tyr	Leu	Gly	Gln	Tyr	Ile	•	
				50					55					60			
ATG	ACT	AAA	CGA	TTA	TAT	GAT	GAG	AAG	CAA	CAA	CAT	ATT	GTA	TAT	TGT		542
												Ile					
			65					70					75				
												AGC					590
Ser	Asn	Asp	Leu	Leu	Gly	Asp	Leu	Phe	Gly	Val	Pro	Ser	Phe	Ser	Val		
		80					85					90					

AAJ	A GAG	G CAG	C AGG	AAA S	ATA	TAT	' ACC	ATC	ATO	TAC	AGG	AAC	TTG	GTA	GTA	638
Lys	s Glu	ı His	s Arg	Lys	Ile	туг	Thi	. Met	Ile	е Туз	Ar	g Asr	ı Le	u Va	l Val	
	95					100					10					
GTC	TAA :	CAC	CAG	GAA	TCA	TCG	GAC	TCA	GGT	ACA	TCT	' GTG	AGT	GAG	AAC	686
Val	Asr	Glr	ı Glm	Glu	Ser	Ser	Asp	Ser	Gly	Thr	Sei	r Val	. Ser	Glu	ı Asn	
110					115					120					125	
AGG	TGT	CAC	CTT	GAA	GGT	GGG	AGT	GAT	CAA	AAG	GAC	CII	GTA	CAA	GAG	734
Arg	Cys	His	Leu	Glu	Gly	Gly	Ser	Asp	Gln	Lys	Asp	Leu	Val	Gln	Glu	
				130					135					140		
CTT	CAG	GAA	GAG	AAA	CCT	TCA	TCT	TCA	CAT	TTG	GTT	TCT	AGA	CCA	TCT	782
Leu	Gln	Glu	Glu	Lys	Pro	Ser	Ser	Ser	His	Leu	Val	Ser	Arq	Pro	Ser	702
			145					150					155			
ACC	TCA	TCT	AGA	AGG	AGA	GCA	ATT	AGT	GAG	ACA	GAA	GAA	AAT	TCA	GAT	830
Thr	Ser	Ser	Arg	Arg	Arg	Ala	Ile	Ser	Glu	Thr	Glu	Glu	Asn	Ser	Asp	930
		160					165					170			vob	
												•				
GAA	TTA	TCT	GGT	GAA	CGA	CAA	AGA	AAA	CGC	CAC	AAA	TCT	GAT	ልርጥ	D TTT	878
Glu	Leu	Ser	Gly	Ġlu	Arg	Gln	Arg	Lys	Arg	His	Lvs	Ser	Asp	Ser	Tla	078
	175					180					185			-		
TCC	CTT	TCC	TIT	GAT	GAA	AGC .	CTG	GCT	CTG	TGT	GTA	ATA .	ልሮር	G N C	እሞኦ	222
Ser	Leu	Ser	Phe	Asp	Glu	Ser	Leu	Ala	Leu	Cvs	Val	Ile	۵۳۰	G1	TIA	926
190					195					200		***	-u.y	GIU		
															205	

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TGT	TGT	GAA	AGA	AGC	AGT	AGC	AGT	GAA	TCT	ACA	GGG	ACG	CCA	TCG	AAT	974
Cys	Cys	Glu	Arg	Ser	Ser	Ser	Ser	Glu	Ser	Thr	Gly	Thr	Pro	Ser	. Asn	
				210					215					220	)	
CCG	GAT	CTT	GAT	GCT	GGT	GTA	AGT	GAA	CAT	TCA	GGT	GAT	TGG	TTG	GAT	1022
Pro	Asp	Leu	Asp	Ala	Gly	Val	Ser	Glu	His	Ser	Gly	Asp	Trp	Leu	Asp	
			225					230					235			
															TCT	1070
Gln	Asp	Ser	Val	Ser	Asp	Gln	Phe	Ser	Val	Glu	Phe	Glu	Val	Glu	Ser	
		240					245					250				
							CIT									1118
Leu	qaA	Ser	Glu	Asp	Tyr	Ser	Leu	Ser	Glu	Glu	Gly	Gln	Glu	Leu	Ser	
	255					260					265					
							CAA									1166
	Glu	Asp	Asp	Glu	Val	Tyr	Gln	Val	Thr	Val	Tyr	Gln	Ala	Gly	Glu	
270					275					280					285	
							GAA									1214
ser	Asp	Thr	Asp		Phe	Glu	Glu	Asp	Pro	Glu	Ile	Ser	Leu	Ala	qaA	
				290					295					300		
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							AAT									1262
ıyr	rrp	rys		Lux	Ser	Сув	Asn	Glu	Met	Asn	Pro	Pro	Leu	Pro	Ser	
			305					310					315			

والاجتمارة المعيهوم بشوراتان

CA	T TG	C AA	C AG	A TGT	r TG	G GC	CIT	r cg:	r gag	G AAT	r TG	G CTI	. cci	GA.	A GAT	1310
Hı	в Су	s As	n Ar	g Cys	s Tr	p Al	a Le	ı Ar	g Gl	u Ası	n Tr	p Le	ı Pro	G1	u Asp	
		32					329					330			-	
AA	A GG	g aa	A GAT	AAA 1	GGG	GAA	ATC	TCI	GAG	AAA	GCC	: AAA	CTG	GAA	AAC	1358
Lys	G1	y Ly	s Asp	Lys	Gl <sub>y</sub>	/ Glu	ı Ile	Se:	r Glu	ı Lya	a Ala	a Lys	. Leu	ı Glı	ı Asn	
	33!					340					349					
TC	A AC	A CAI	A GCT	' GAA	GAG	GGC	TTT	GAT	GTT	CCT	GAT	TGT	AAA	AAA	ACT	1406
Ser	Thi	Glr	n Ala	Glu	Glu	Gly	Phe	Asp	Val	. Pro	Ası	Cys	Lys	Lys	Thr	
350	•				355					360				-	365	
ATA	GTG	TAA	GAT	TCC	AGA	GAG	TCA	TGT	GTT	GAG	GAA	AAT	GAT	GAT	AAA	1454
Ile	Val	Asn	Asp	Ser	Arg	Glu	Ser	Cys	Val	Glu	Glu	Asn	Asp	Asp	Lvs	
				370					375				_	380		
ATT	ACA	CAA	GCT	TCA	CAA	TCA	CAA	GAA	AGT	GAA	GAC	TAT	TCT	CAG	CCA	1502
Ile	Thr	Gln	Ala	Ser	Gln	Ser	Gln	Glu	Ser	Glu	Asp	Tyr	Ser	Gln	Pro	
			385					390					395			
TCA	ACT	TCT	AGT	AGC	ATT	ATT	TAT	AGC	AGC	CAA	GAA	GAT	GTG .	AAA	GAG	1550
Ser	Thr	Ser	Ser	Ser	Ile	Ile	Tyr	Ser	Ser	Gln	Glu	Asp	Val	Lvs	Glu	2330
		400					405					410		•		
TIT	GAA	AGG	GAA	GAA .	ACC	CAA	GAC .	AAA	GAA	GAG .	AGT	GTG (	GAA 1	rcr .	AGT	1598
Phe	Glu	Arg	Glu	Glu	Thr	Gln	Asp	Lys	Glu	Glu	Ser	Val	Glu	Ser	Ser	
	415					420					425					

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	CCC	C11	AAI	GCC	ATT	GAA	CCT	TGT	GTG	ATT	TGT	CAA	GGT	CGA	ccr	1646
Leu	Pro	Leu	Asn	Ala	Ile	Glu	Pro	Cys	Val	Ile	Сув	Gln	Gly	Arg	Pro	
430					435					440					445	
AAA	AAT	GGT	TGC	ATT	GTC	CAT	GGC	AAA	ACA	GGA	CAT	CTT	ATG	GCC	TGC	. 1694
Lys	Asn	Gly	Cys	Ile	Val	His	Gly	Lys	Thr	Gly	His	Leu	Met	Ala	Сув	
				450					455					460		
			GCA													1742
Phe	Thr	Cys	Ala	Lys	Lys	Leu	Lys	Lys	Arg	Asn	Lys	Pro	Сув	Pro	Val	
			465					470					475			
			CCA													1784
Сув	Arg	Gln	Pro	Ile	Gln	Met	Ile	Val	Leu	Thr	Tyr	Phe	Pro			
		480					485					490				
TAGT	TGA	CT C	TCTA	TAAG	A GA	ATTA	ATAT.	TIT	CTAA	CTA	TATA	ACCC	TA G	GAAT	TTAGA	1844
					а та	TATC	aaag	TGA	.GAAA	ATG	ccrc	AATT	CA C	ATAG	ATTTC	1904
CAAC	'CTG#	laa t	TTAT	TCAC												1904
CAAC	'CTG#	laa t	TTAT	TCAC											ATTTC	1904 1964
CAAC	CTT	aa t	TATT	TCAC TTGA	C CT.	ACTT	TGGT	AGT	GGAA	TAG '	TGAA	TACT	TA C	TATAI	ATTTG	
CAAC	CTT	aa t	TATT	TCAC TTGA	C CT.	ACTT	TGGT	AGT	GGAA	TAG '	TGAA	TACT	TA C	TATAI		
TTCT ACTT	CTGI CTTT	AAA T PAG T	TTAT ATAA TAGC	TCAC TTGA TCAT	c cr.	ACTT TTAC	TGGT ACCA	AGT	GGAA CCTA	TAG	TGAA TTAA	TACT ATAA	IA C	TATAI	ATTTG TCTGT	1964 2024
TTCT ACTT	CTGI CTTT	AAA T PAG T	TTAT ATAA TAGC	TCAC TTGA TCAT	c cr.	ACTT TTAC	TGGT ACCA	AGT	GGAA CCTA	TAG	TGAA TTAA	TACT ATAA	IA C	TATAI	ATTTG	1964 2024
CAAC TTCT ACTT	CTGA CTTT GAAT	AAA T TAG T TAT G	TTAT ATAA TAGC	TCAC TTGA TCAT	C CT C CT G TT	ACTT TTAC	TGGT ACCA	ACT	GGAA CCTA AAAT	TAG	TGAA TTAA TATA	TACT ATAA' TGAC	TA CT	TATAI CTAC: TAAA1	ATTTG FCTGT FGTAA	1964 2024 2084
CAAC TTCT ACTT	CTGA CTTT GAAT	AAA T TAG T TAT G	TTAT ATAA TAGC	TCAC TTGA TCAT	C CT C CT G TT	ACTT TTAC	TGGT ACCA	ACT	GGAA CCTA AAAT	TAG	TGAA TTAA TATA	TACT ATAA' TGAC	TA CT	TATAI CTAC: TAAA1	ATTTG TCTGT	1964 2024 2084
CAAC TTCT ACTT CTTA	CTTI GAAT AATG	AAA T TAT G	TTAT TAGC	TCAC TTGA TCAT CTTG	C CT C CT G TT	ACTT TTAC	TGGT ACCA FFFT	ACT CTT	GGAA CCTA AAAT GTTA	ATT	TGAA TTAA TATA	TACT ATAA' TGACI	TA CT	TATAI CTAC: TAAA1 CAGTO	ATTTG  TCTGT  TGTAA  GGGTG	1964 2024 2084

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2264

2372

CCCAATTAGC TTGGCCTACA GTCATCTGCC ACCACACCTG GCTAATTTTT TGTACTTTTA GTAGAGACAG GGTTTCACCG TGTTAGCCAG GATGGTCTCG ATCTCCTGAC CTCGTGATCC GCCCACCTCG GCCTCCCAAA GTGCTGGGAT TACAGGCATG AGCCACCG (2) INFORMATION FOR SEQ ID NO:3: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 491 amino acids (B) TYPE: amino acid (D) TOPOLOGY: linear (ii) MOLECULE TYPE: protein (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3: Met Cys Asn Thr Asn Met Ser Val Pro Thr Asp Gly Ala Val Thr Thr 1 5 10 15 Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro 20 25 30 Leu Leu Lys Leu Lys Ser Val Gly Ala Gln Lys Asp Thr Tyr 35

Thr Met Lys Glu Val Leu Phe Tyr Leu Gly Gln Tyr Ile Met Thr Lys 55 60

50

40

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Arg	Leu	Tyr	Asp	Glu	Lys	Gln	Gln	His	Ile	Val	Tyr	Сув	Ser	Asn	Asp	
65					70					75					80	
Leu	Leu	Gly	Asp	Leu	Phe	Gly	Val	Pro	Ser	Phe	Ser	Val	Lvs	Glu	His	
				85					90				-4 -	95		
Arg	Lys	Ile	Tyr	Thr	Met	Ile	Tyr	Arg	Asn	Leu	Val	Val	Val	Asn	Gln	
			100					105					110		<b></b>	
Gln	Glu	Ser	Ser	Asp	Ser	Gly	Thr	Ser	Val	Ser	Glu	Asn	Δνα	Cre	ui e	
		115		_		-	120					125	AL Y	CyB	ure	
												145				
Leu	Glu	Gly	Gly	Ser	Asp	Gln	Lvs	Asn	Leu	Va 1	Gl n	Glu	7	<b>01</b> =	<b>61</b>	
	130	•				135	~, 5	vob	Den	Val		GIU	Leu	GIN	GIU	
						-33					140					
Glu	Lvs	Pro	Ser	Ser	Ser	Hia	T.au	Wa 1	50=	N	D	Ser		_	_	
145					150		Dea	Val	Ser		PIO	ser	Inr	Ser		
					130					155					160	
Ara	Ara	Ara	בו ב	Tla	Sar	C1	Th	G1	<b>a</b> 1	_						
-12 y	Arg	Arg	710	165	261	GIU	Inr	GIU		Asn	Ser	Asp	Glu	Leu	Ser	
				165					170					175		
21,,	Gl u	۸	C1 =	N	*	•		_	_							
JIY	GIU	<b>A</b> 4 9		Arg	Lys	Arg	nıs		Ser	Asp	Ser	Ile	Ser	Leu	Ser	
			180					185					190			
7h ~	<b>&gt;</b>	<b>G1</b>		_		_	_									
rne	Asp		ser	Leu	Ala	Leu		Val	Ile	Arg	Glu	Ile	Сув	Сув	Glu	
		195					200					205				
	_															
		Ser	Ser	Ser	Glu	Ser	Thr	Gly	Thr	Pro	Ser	Asn	Pro	Asp	Leu	
	210					215					220					

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Ası	p Al	a Gl	y Va	l Se	r Glu	His	s Se	r Gl	y Ası	o Tr	Le	ı Ası	Glr	a Asj	Se:
229	5				230					235	5				24
Va:	l Se	r As	p Gl	n Phe	e Ser	Val	. Glu	ı Phe	e Glu	ı Val	. Glu	ı Ser	Leu	ı Ası	Se:
				249					250					255	
															•
Glu	Asp	ту	r Se	r Let	ı Ser	Glu	Glu	ı Gly	/ Glm	ı Glu	Leu	Ser	. Nac	. 61.	
			26					265					270		. Ast
													270	'	
Asp	Glu	. Va	l Ty:	r Glr	Val	Thr	Vaí	ጥተታ	. 615		<b>0</b> 1.				
		27					280		GIII	Ala	GIY		Ser	Asp	Thr
							260	'				285			
Aso	Ser	- Dha	a (3)		<b>&gt;</b>										
- 10 p	290		- 010	ı Giü	Asp		Glu	Ile	Ser	Leu	Ala	Ąsp	Tyr	Trp	Lys
	230					295					300				
_		_													
		Ser	с Сув	Asn	Glu	Met	Asn	Pro	Pro	Leu	Pro	Ser	His	Сув	Asn
305					310					315					320
Arg	Cys	Trp	Ala	Leu	Arg	Glu	Asn	Trp	Leu	Pro	Glu	Asp	Lys	Gly	Lvs
				325					330				-	335	
Asp	Lys	Gly	Glu	Ile	Ser	Glu	Lys	Ala	Lys	Leu	Glu	Δan	Ser	Th-	C1-
			340					345	•			*****		1111	GIII
													350		
Ala	Glu	Glu	Gly	Phe	Asp	Va1	Pro	Δen	C) ra	T	•	<b></b>			
		355	•	_				veb	Cys	ràs	Lys		Ile	Val	Asn
		- 3					360					365			
Asp	Ser	۵~ς	G1	Cor	<b>0</b>										
P		~r y	GIU	ser	Сув		Glu	Glu	Asn	Asp	Asp	Lys	Ile	Thr	Gln
	370					375					380				

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Ala Ser Gln Ser Gln Glu Ser Glu Asp Tyr Ser Gln Pro Ser Thr Ser 385

Ser Ser Ile Ile Tyr Ser Ser Gln Glu Asp Val Lys Glu Phe Glu Arg

Glu Glu Thr Gln Asp Lys Glu Glu Ser Val Glu Ser Ser Leu Pro Leu
420 425 430

Asn Ala Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro Lys Asn Gly
435
440
445

Cys Ile Val His Gly Lys Thr Gly His Leu Met Ala Cys Phe Thr Cys
450
455
460

Ala Lys Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val Cys Arg Gln
465 470 475 480

Pro Ile Gln Met Ile Val Leu Thr Tyr Phe Pro

#### (2) INFORMATION FOR SEQ ID NO:4:

#### (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1710 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

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wu	y.)	/ Z	IJZ		a

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(ii) MOLECULE TYPE: cDNA	
(iii) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: NO	
(V1) ORIGINAL SOURCE:  (A) ORGANISM: Mus musculus	
(ix) FEATURE:	
(A) NAME/KEY: CDS (B) LOCATION: 2021668	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:	
GAGGAGCCGC CGCCTTCTCG TCGCTCGAGC TCTGGACGAC CATGGTCGCT CAGGCCCCGT	60
CCGCGGGGCC TCCGCGCTCC CCGTGAAGGG TCGGAAGATG CGCGGGAAGT AGCAGCCGTC	120
TGCTGGGCGA GCGGGAGACC GACCGGACAC CCCTGGGGGGA CCCTCTCGGA TCACCGCGCT	180
TCTCCTGCGG CCTCCAGGCC A ATG TGC AAT ACC AAC ATG TCT GTG TCT ACC  Met Cys Asn Thr Asn Met Ser Val Ser Thr	231

GAG GGT GCT GCA AGC ACC TCA CAG ATT CCA GCT TCG GAA CAA GAG ACT

Glu Gly Ala Ala Ser Thr Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr

15

20
25

5

10

1

CTG	GTI	AGA	CCA	AAA .	CCA	TTG	CTT	TTG	AAG	TTG	TTA	AAG	TCC	GTI	GGA	327
Leu	Val	Arg	Pro	Lys	Pro	Leu	Leu	Let	Lys	Leu	Le.	ı Lyı	s Se	r Va	l Gly	
			30					35					4			
GCG	CAA	AAC	GAC	ACT	TAC	ACT	ATG	AAA	GAG	ATT	ATA	TIT	TAT	' ATT	GGC	375
Ala	Gln	Asn	qaA	Thr	Tyr	Thr	Met	Lys	Glu	Ile	Ile	Phe	ту:	r Ile	e Gly	
		45					50					55				
CAG	TAT	ATT	ATG	ACT	AAG	AGG	TTA	TAT	GAC	GAG	AAG	CAG	CAG	CAC	ATT	423
Gln	Tyr	Ile	Met	Thr	Lys	Arg	Leu	Tyr	Asp	Glu	Lys	Glr	Glr	ı His	: Ile	
	60					65					70					
GTG	TAT	TGT	TCA	AAT	GAT	CTC	CTA	GGA	GAT	GTG	TTT	GGA	GTC	CCG	AGT	471
Val	Tyr	Cys	Ser	Asn	Asp	Leu	Leu	Gly	Asp	Val	Phe	Gly	Val	Pro	Ser	
75					80					85					90	
TTC	TCT	GTG	AAG	GAG	CAC	AGG	AAA	ATA	TAT	GCA	ATG	ATC	TAC	AGA	AAT	519
Phe	Ser	Val	Lys	Glu	His	Arg	Lys	Ile	Tyr	Ala	Met	Ile	Tyr	Arg	Asn	
				95					100					105		
TTA	GTG	GCT	GTA	AGT	CAG	CAA	GAC	TCT	GGC	ACA	TCG	CTG	AGT	GAG	AGC	567
Leu	Val	Ala	Val	Ser	Gln	Gln	Asp	Ser	Gly	Thr	Ser	Leu	Ser	Glu	Ser	
			110					115					120			
AGA	CGT	CAG	CCT	GAA	GGT	GGG	AGT (	GAT	CTG .	AAG (	GAT	CCT	TTG	CAA	GCG	615
Arg	Arg	Gln	Pro	Glu	Gly	Gly	Ser	Asp	Leu	Lys	Asp	Pro	Leu	Gln	Ala	
		125					130					135				

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CCA CCA GAA GAG AAA CCT TCA TCT TCT GAT TTA ATT TCT AG	A CTG TCT 66
Pro Pro Glu Glu Lys Pro Ser Ser Ser Asp Leu Ile Ser A	FG Leu Ser
140 145 150	, 502
ACC TCA TCT AGA AGG AGA TCC ATT AGT GAG ACA GAA GAG AA	C ACA GAT 711
Thi Ser Ser Arg Arg Arg Ser Ile Ser Glu Thr Glu Glu A	on Thr Asp
155 160 165	170
GAG CTA CCT GGG GAG CGG CAC CGG AAG CGC CGC AGG TCC CT	G TCC TTT 759
Glu Leu Pro Gly Glu Arg His Arg Lys Arg Arg Arg Ser Le	u Ser Phe
175 180	185
GAT CCG AGC CTG GGT CTG TGT GAG CTG AGG GAG ATG TGC AGG	
Asp Pro Ser Leu Gly Leu Cys Glu Leu Arg Glu Met Cys Se	GGC GGC 807
190 195 20	
ACG AGC AGC AGC AGC AGC AGC GAG TCC ACA GAG ACG	CCC TCG 855
Thr Ser Ser Ser Ser Ser Ser Ser Glu Ser Thr Glu Th	r Pro Ser
205 210 215	
CAT CAG GAT CTT GAC GAT GGC GTA AGT GAG CAT TCT GGT GAT	Tree eme
His Gln Asp Leu Asp Asp Gly Val Ser Glu His Ser Gly Asp	rgc crg 903
220 225 230	cys Leu
GAT CAG GAT TCA GTT TCT GAT CAG TTT AGC GTG GAA TTT GAA	CTT CLC
Asp Gln Asp Ser Val Ser Asp Gln Phe Ser Val Glu Phe Glu	Val Glu
235 240 245	250

TCT	CTC	GAC	TCG	GAA	GAT	TAC	AGC	CTG	AGI	GAC	GAZ	A GG	G CA	C GA	G CTC	99
Ser	Leu	ı Asp	Ser	Glu	Asp	Туг	Ser	Leu	Se	r Ası	Gl	u Gl	y Hi	s Gl	u Lei	1
				255					260					26		
TCA	GAT	' GAG	GAT	GAT	GAG	GTC	TAT	CGG	GTC	ACA	GTC	TAT	CAC	AC	A GGA	104
Ser	qaA	Glu	Asp	Asp	Glu	Val	Tyr	Arg	Va]	. Thr	. Va	l Ty:	r Gl	n Th	r Gly	•
			270					275					28			
GAA	AGC	GAT	ACA	GAC	TCT	TTT	GAA	GGA	GAT	CCT	GAG	ATI	TCC	TTA	GCT	1095
Glu	Ser	Asp	Thr	Asp	Ser	Phe	Glu	Gly	Asp	Pro	Glu	ı Ile	e Se	r Le	u Ala	
		285					290					295				
GAC	TAT	TGG	AAG	TGT	ACC	TCA	TGC	AAT	GAA	ATG	AAT	CCT	ccc	CII	CCA	1143
Asp	Tyr	Trp	Lys	Сув	Thr	Ser	Сув	Asn	Glu	Met	Asn	Pro	Pro	Le	ı Pro	
	300					305					310					
TCA	CAC	TGC	AAA	AGA	TGC	TGG	ACC	CIT	CGT	GAG	AAC	TGG	CTT	CCA	GAC	1191
Ser	His	Сув	Lys	Arg	Сув	Trp	Thr	Leu	Arg	Glu	Asn	Trp	Leu	Pro	Asp	
315					320					325					330	
GAT	AAG	GGG	AAA	GAT	AAA	GTG	GAA	ATC	TCT	GAA	AAA	GCC	AAA	CTG	GAA	1239
qaA	Lys	Gly	Lys	Asp	Lys	Val	Glu	Ile	Ser	Glu	Lys	Ala	Lys	Leu	Glu	
				335					340				•	345		
AC '	TCA	GCT	CAG	GCA (	GAA (	GAA (	GGC '	TTG (	GAT	GTG	CCT	GAT	GGC	AAA	AAG	1287
sn	Ser	Ala	Gln	Ala	Glu	Glu	Gly	Leu	Asp	Val	Pro	Asp	Glv	Lve	Lys	
			350					355				_	360	_, •	-, 3	

CTG ACA GAG A	AT GAT GCT AAA	GAG CCA TGT GCT G	AG GAG GAI' AGC GAG	
Leu Thr Glu Ag	sn Asp Ala Lys	Glu Pro Cys Ala G	lu Glu Asp Ser Glu	1335
365		370	375	
GAG AAG GCC GA Glu Lys Ala Gl	A CAG ACG CCC	CTG TCC CAG GAG AG	T GAC GAC TAT TCC	1383
380	385	39		
CAA CCA TCG ACGIN Pro Ser Th	T TCC AGC AGC	ATT GTT TAT AGC AGG	C CAA GAA AGC GTG	1431
373	400	405	410	
Lys Glu Leu Lys	S Glu Glu Thr	CAG CAC AAA GAC GAG Gln His Lys Asp Glu 420	ı Ser Val Glu Ser	1479
AGC TTC TCC CTG	AAT GCC ATC G	GAA CCA TGT GTG ATC	TGC CAG GGG CGG	1527
430		435	440	
Pro Lys Asn Gly	Cys Ile Val H	AC GGC AAG ACT GGA	CAC CTC ATG TCA	1575
TGT TTC ACG TGT		TA AAA AAA AGA AAC	455	
Cys Phe Thr Cys	Ala Lys Lys L	eu Lys Lys Arg Asn	AAG CCC TGC CCA	1623
460	465	470	-,	

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GTG TGC AGA CAG CCA ATC CAA ATG ATT GTG CTA AGT TAC TTC AAC

Val Cys Arg Gln Pro Ile Gln Met Ile Val Leu Ser Tyr Phe Asn

475

480

485

TAGCTGACCT GCTCACAAAA ATAGAATTTT ATATTTCTAA CT

- (2) INFORMATION FOR SEQ ID NO:5:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 489 amino acids
    - (B) TYPE: amino acid
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: protein
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Cys Asn Thr Asn Met Ser Val Ser Thr Glu Gly Ala Ala Ser Thr

1 5 10 15

Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro

Leu Leu Lys Leu Lys Ser Val Gly Ala Gln Asn Asp Thr Tyr
35 40 45

Thr Met Lys Glu Ile Ile Phe Tyr Ile Gly Gln Tyr Ile Met Thr Lys
50 55 60

Ar	g Le	ŧu	Ty:	r As	p Gl	u Ly	B Gl	n Gl	n Hi	s Ile	e Val	l Tyr	Сув	Ser	Asr	Asp
6	5					7					75		-			
																80
Lei	ı Le	·u	Glv	/ Ası	o Vai	l Ph	a (3)	, 17-1	1 D							
			•				- 01)	y va.	L PIC	) Ser	Phe	Ser	Val	Lys	Glu	His
					85	•				90	)				95	
Arg	J Ly	8	Ile	Туз	Ala	Me t	: Ile	Туг	Arg	Asn	Leu	Val	Ala	Val	Ser	Gln
				100					105					110		
														0		
Glr	As	p	Ser	Gly	Thr	Ser	Leu	Ser								Gly
			115							ser	Arg	Arg	Gln	Pro	Glu	Gly
			- 4 3					120					125			
Gly	Se:	r	Asp	Leu	Lys	Asp	Pro	Leu	Gln	Ala	Pro	Pro	Glu	Glu	Lys	Pro
	13	0					135					140			-	
Ser	Se	r :	Ser	Asp	Leu	Tle	Sar	۸~~	T ~	0	Thr					
145							261	Arg	Leu	ser	Thr	Ser	Ser	Arg	Arg	Arg
						150					155					160
Ser	Ile	2	Ser	Glu	Thr	Glu	Glu	Asn	Thr	Asp	Glu	Leu	Pro	Gly	Glu	Ara
					165					170				-	175	
															-,,	
His	Arg	I	ув	Arg	Arg	Ara	Ser	T.a.ı	°-2	Dh.a	Asp	_	_			
			-	180	3	3	561	nea		rne	Asp	Pro	Ser	Leu	Gly	Leu
				100					185					190		
Cys	Glu	L	eu	Arg	Glu	Met	Сув	Ser	Gly	Gly	Thr	Ser	Ser	Ser	Ser	Ser
			95					200					205			
Ser	Ser	s	er	Glu	Ser	Thr	Glu	Th∽	Dro	Ca	His		_			
	210			_	<b>-</b>			-111	110	ser	nis	Gln .	Asp :	Leu .	Asp .	Asp
	-10						215					220				

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Gly	y Va	1 S	er	Glu	ı Ri	s Se:	r Gly	y Asp	с Суя	Lev	ı Asp	Gln	Asp	Ser	Val	Ser
225						230					235		•			240
																240
Asŗ	G1:	n Ph	ıe	Ser	. Vai	l Glu	ı Phe	e Glu	ı Val	Gli	ı Ser	Leu	Asn	Ser	Glu	yen
					249					250			· ·····p	001		Asp
															255	
Tyr	: Se	r Le	u.	Ser	as,	Glu	ı Gly	7 His	Glu	Leu	Ser	Aso	Glu	Ann	Agn	Gl.,
				260					265			- &-		270	710p	014
														2,0		
Val	Туз	Ar	g '	Val	Thr	. Val	Tyr	Gln	Thr	Glv	Glu	Ser	Asn	Th∽	) an	Co=
		27						280		•			285	****	vaħ	Set
													403			
Phe	Glu	G1	y 1	qaA	Pro	Glu	Ile	Ser	Leu	Ala	Asp	Tur	Trans.	****	~	(Tibe est
	290						295				Пор	300	110	Lyb	Сув	inr
												300				
Ser	Суз	As	n C	3lu	Met	Asn	Pro	Pro	Leu	Pro	Ser	W-1	<b></b>	•	_	_
305						310						uis	Сув	rys	Arg	
											315					320
Trp	Thr	Le	<b>ב</b> ב	Lra	Glu	Asn	Trn	Leu	Dro	N	Asp	_				
					325			Dea	PIO		Asp	rys	Gly			Lys
					323					330					335	
Val	Glu	Ile		er	Glu	Laza	ת א	<b>7</b>	• -							
				40	<b>51</b> u	Dy B	ATG	Lys		Glu	Asn	Ser	Ala	Gln	Ala	Glu
			,	40					345					350		
G1	G1	T			17- 3	_	_									
Giu	GIY			ga	Val	Pro	Asp	Gly	Lys	Lys	Leu	Thr	Glu	Asn .	qaA	Ala
		355	•					360					365			
_																
Lys		Pro	C .	ys	la	Glu	Glu	qaA	Ser	Glu	Glu	Lys	Ala	Glu (	Gln '	Thr
	370						375					380				

- 54 -

Pro Leu Ser Gln Glu Ser Asp Asp Tyr Ser Gln Pro Ser Thr Ser Ser 385

Ser Ile Val Tyr Ser Ser Gln Glu Ser Val Lys Glu Leu Lys Glu Glu 405 410 415

Thr Gln His Lys Asp Glu Ser Val Glu Ser Ser Phe Ser Leu Asn Ala
420
425
430

Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro Lys Asn Gly Cys Ile
435
440
445

Val His Gly Lys Thr Gly His Leu Met Ser Cys Phe Thr Cys Ala Lys
450 455 460

Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val Cys Arg Gln Pro Ile 465 470 480

Gln Met Ile Val Leu Ser Tyr Phe Asn 485

PCT/US93/03199 WO 93/20238

-55-International Application No: PCT/ /

MICROORGANISMS						
Optional Short in connection with the microorganism referred to an page						
A. IDENTIFICATION OF DEPOSIT !						
Further deposits are identified on an additional sheet 2 s						
Name of depository inetitution 4						
AMERICAN TYPE ON THRE COLLECTION	,					
AMERICAN TYPE CULTURE COLLECTION						
Address of depositary institution (including postal code and country	12301 Parklawn Drive Rockville, Maryland 20852 United States of America					
Date of deposit *	Accession Number 4					
March 11, 1993	HB 11290					
B. ADDITIONAL INDICATIONS! (leave blank if not applicable	). This information is continued on a separate attached short					
is sought a sample of the deposit available until the publication of European patent or until the date refused or withdrawn or is deemed of such a sample to an expert nom the sample. (Rule 28(4) EPC)  C. DESIGNATED STATES FOR WHICH INDICATIONS AR	on which the application has been to be withdrawn, only the issue inated by the person requesting  E MADE: (M the indications are not for all decolorated States)					
D. SEPARATE FURNISHING OF INDICATIONS 4 (leave bla	nk if not applicable)					
- Accession Number of Copped 7	al Bursau later * (Specify the general nature of the Indications + 8					
E. This sheet was received with the international application	(Authorized Officer)					
The date of receipt (from the applicant) by the internation	al Bureau 10					
<b>***</b>	(Authorized Officer)					

Form PCT/RO/134 (January 1981)

International Application No: PCT/

	CROORGANISMS
Optional Sheet in connection with the microorganism re	eferred to an page 10 line 19 of the description t
A. IDENTIFICATION OF DEPOSIT	of the description 1
Further deposits are identified on an additional sheet	• <u>(</u> [] •
Name of depositary institution *	
AMERICAN TYPE CULTURE COLLEC	CTION
Address of depositary institution (including postal code 12301 Parklawn Drive	end country) •
Rockville, Maryland 20852, U	ΔΖΙ
March 11, 1993	Accession Number • HB 11290
S. ADDITIONAL INDICATIONS ! (leave blank if no	et applicable). This information is continued on a separate attached sheet
IF2 - Hybridoma	
C. DESIGNATED STATES FOR WHICH INDICAT	IOMS ARE MADE : (If the indications are not for all designated States)
D. SEPARATE FURNISHING OF INDICATIONS	(leave blank if not applicable)
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"Accession Number of Deposit ")	national bureau later * (Specify the general nature of the indications e.g.,
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	(Authorized Officer)

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	International Application No: PCT/ /
MICRO	PRGANISMS
Optional Sheet in connection with the microorganism referred to	an ease 10 the description t
A. IDENTIFICATION OF DEPOSIT	
Further deposits are identified on an additional sheet	
Name of depositary institution 4	
AMERICAN TYPE CULTURE COLLECTION	l
Address of depository institution (including postal code and could 12301 Parklawn Drive Rockville, Maryland 20852, USA	entry) *
ROCKVITTE, Mary rand 20002, OSA	
March 11, 1993	Accession Number • HB 11291
8. ADDITIONAL INDICATIONS ! (leave blank if not applic	sale). This information is continued on a separate attached sheet
ED9 - Hybridoma	
C. DESIGNATED STATES FOR WHICH INDICATIONS	ARE MADE * (If the indications are not for all designated States)
D. SEPARATE FURNISHING OF INDICATIONS ! (loave	s blank if not applicable)
The indications listed below will be submitted to the Internet "Accession Number of Deposit")	tional Bureau leter <sup>4</sup> (Specify the general nature of the indications e.g.,
E. This sheet was received with the international applicati	tion when filed (to be checked by the receiving Office)
	M. Wilmes (Authorized Officer)
The date of receipt (from the applicant) by the Internal	dional Suracy 19
-45	(Authorized Officer)

#### **CLAIMS**

- 1. A method of diagnosing a neoplastic tissue in a human comprising:
  detecting amplification of human MDM2 gene or elevated expression of a
  human MDM2 gene product in a tissue or body fluid isolated from a human, wherein
  amplification of the human MDM2 gene or elevated expression of human MDM2 gene
  product provides a diagnosis of neoplasia or the potential for neoplastic development.
  - 2. The method of claim 1 wherein gene amplification is detected.
- 3. The method of claim 1 wherein elevated expression of a gene product is detected, said gene product being mRNA.
- 4. The method of claim 1 wherein elevated expression of a gene product is detected, said gene product being human MDM2 protein.
- 5. The method of claim 3 wherein said mRNA is detected by Northern blot analysis by hybridizing mRNA from said tissue to a human MDM2 nucleotide probe.
- 6. The method of claim 5 wherein the human MDM2 nucleotide probe comprises nucleotides 1-2372 of human MDM2, as shown in Figure 1, or fragments thereof consisting of at least 14 contiguous nucleotides.
- 7. The method of claim 4 wherein human MDM2 protein is detected by Western Blot analysis by reacting human MDM2 proteins with antibodies which are immunospecific for MDM2 protein.
- 8. The method of claim 2 wherein the gene amplification is detected using polymerase chain reaction.
- 9. The method of claim 2 wherein amplification of the human MDM2 gene is detected by Southern blot analysis wherein the human MDM2 gene is hybridized with a nucleotide probe which is complementary to hMDM2 DNA.
- 10. The method of claim 2 wherein gene amplification is determined by comparing the copy number of hMDM2 in the tissue to the copy number of hMDM2 in a normal tissue of the human.

- 11. The method of claim 3 wherein elevated expression of mRNA is determined by comparing the amount of hMDM2 mRNA in the tissue to the amount of hMDM2 mRNA in a normal tissue of the human.
- 12. The method of claim 4 wherein elevated expression of hMDM2 protein is determined by comparing the amount of hMDM2 protein in the tissue to the amount of hMDM2 protein in a normal tissue of the human.
- 13. The method of claim 2 wherein gene amplification is detected when at least 3-fold more hMDM-2 DNA is observed in the tissue relative to a control sample comprising a normal tissue.
- 14. The method of claim 3 wherein elevated expression is detected when at least 3-fold more hMDM-2 mRNA is observed in the tissue relative to a control sample comprising a normal tissue.
- 15. The method of claim 4 wherein elevated expression is detected when at least 3-fold more hMDM2 protein is observed in the tissue relative to a control sample comprising a normal tissue.
  - 16. The method of claim 1 wherein the neoplasia is a sarcoma.
- 17. The method of claim 16 wherein the sarcoma is a liposarcoma, malignant fibrous histiocytoma, or osteosarcoma.
- 18. A cDNA molecule comprising nucleotides 1 to 2372, as shown in Figure 1, or fragments thereof, consisting of at least 14 contiguous nucleotides.
- 19. The cDNA molecule of claim 18 comprising the coding sequence of human MDM2.
  - 20. Human MDM2 protein substantially free of other human proteins.
- 21. A preparation of antibodies specifically immunoreactive with human MDM2 protein.
  - 22. The preparation of claim 21 wherein the antibodies are monoclonal antibodies.
- 23. A nucleotide probe comprising a sequence of at least 10 nucleotides which are complementary to nucleotides 1-2372 of human MDM2 gene, as shown in Figure 1.

- 24. A kit for detecting the amplification of a human MDM2 gene in a human tissue or body fluid sample comprising: a nucleic acid probe capable of hybridizing to said human MDM2 gene under conditions of high stringency, and instructions for determining said amplification.
- 25. A kit for detecting elevated expression of a human MDM2 mRNA in a human tissue or body fluid sample comprising: a nucleic acid probe capable of hybridizing to said mRNA, and written instructions for determining elevated expression of mRNA.
- A kit for detecting elevated expression of a human MDM2 protein in a human tissue or body fluid sample comprising MDM2 protein-specific antibodies and written instructions for determining elevated expression of human MDM2 protein.
- 27. A method of treating a neoplastic cell or a cell having neoplastic potential, comprising:

administering to a cell a therapeutically effective amount of an inhibitory compound which interferes with the expression of human MDM2 gene.

- 28. The method of claim 27 wherein expression of the human MDM2 gene is inhibited by administering antisense oligonucleotides.
- 29. The method of claim 27 wherein expression of the human MDM2 gene is inhibited by administering triple-strand forming oligonucleotides which interact with DNA.
- 30. A method for identifying compounds which interfere with the binding of human MDM-2 to human p53, comprising:

binding a predetermined quantity of a first human protein which is detectably labelled to a second human protein;

adding a compound to be tested for its capacity to inhibit binding of said first and second proteins to each other;

determining the quantity of the first human protein which is displaced from or prevented from binding to the second human protein;

wherein the first human protein is MDM-2 and the second human protein is p53 or the first human protein is p53 and the second human protein is MDM-2.

- 31. The method of claim 30 wherein one of said two human proteins is fixed to a solid support.
- 32. The method of claim 30 wherein an antibody specifically immunoreactive with said second human protein is used to separate first human protein bound first human protein.
- 33. A method for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification, comprising:

administaring a polypeptide to tumor cells which contain a human MDM2 gene amplification, said polyptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide being capable of binding to human MDM2.

- 34. The method of claim 33 wherein said polypeptide comprises amino acids 1-41 of p53.
- 35. The method of claim 33 wherein said polypeptide comprises amino acids 13-57 of p53.
- 36. The method of claim 33 wherein said polypeptide comprises amino acids 1-50 of p53.
- 37. A method for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification, comprising:

administering to tumor cells which contain a human MDM2 gene amplification a DNA molecule which expresses a polypeptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide being capable of binding to human MDM2.

38. The method of claim 37 wherein said polypeptide comprises amino acids 1-41 of p53.

- 39. The method of claim 37 wherein said polypeptide comprises amino acids 13-57 of p53.
- 40. The method of claim 37 wherein said polypeptide comprises amino acids 1-50 of p53.
- 41. A polypeptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide capable of binding to human MDM2.
  - 42. The polypeptide of claim 41 which comprises amino acids 1-41 of p53.
  - 43. The polypeptide of claim 41 which comprises amino acids 13-57 of p53.
  - 44. The polypeptide of claim 41 which comprises amino acids 1-50 of p53.
- 45. The preparation of claim 21 wherein the antibodies do not bind to other human proteins.
- 46. The preparation of claim 21 wherein the antibodies do not bind to human proteins of M<sub>r</sub> 75-85K, 105-120K, and 170-200K.
- 47. The preparation of claim 21 wherein the antibodies bind to the epitope bound by antibodies secreted by hybridoma IF2 (ATCC HB 11290).
- 48. The preparation of claim 21 wherein the antibodies bind to the epitope bound by antibodies secreted by hybridoma ED9 (ATCC HB 11291).
- 49. The method of claim 7 wherein the antibodies bind to the epitope on hMDM2 bound by antibodies secreted by hybridoma IF2 (ATCC HB 11290).
- 50. The method of claim 4 wherein human MDM2 protein is detected by immunohistochemistry.
- 51. The method of claim 50 wherein antibodies are employed in the immunohistochemistry which bind to an epitope on hMDM2 bound by the antibodies secreted by ED9 (ATCC HB 11291).
- 52. The method of claim 50 wherein antibodies are employed in the immunohistochemistry which bind to an epitope on hMDM2 bound by the antibodies secreted by IF2 (ATCC HB 11290).

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- 53. The method of claim 4 wherein human MDM2 protein is detected by immunoprecipitation.
- 54. A hybridoma cell having the identifying characteristics of ED9 (ATCC HB 11291).
- 55. A hybridoma cell having the identifying characteristics of IF2 (ATCC HB 11290).

# FIG. IA(I)

1	GCACCGCGCGAGCTTGGCTGCTTCTGGGG
84	* AG GGCCGCGACCCCTCTGACCGAGATCCTGCTC
168	CGT GC GG CTCCGCGCTCCCCG GAAC GTGCCCTGGCCCGGAGAGTGGAATGATCCCC
252 1	ACC GACACCCCTGGGGGACC TCG AT GGAGTCTTGAGGGACCCCGACTCCAAGCG
336 9	T C G C G CCTACTGATGGTGCTGTAACCACCTCACAGA P T D G A V T T S Q S E A S
<b>420</b> 37	
504 65	
588 93	G A GTGAAAGAGCACAGGAAAATATATACCATGA V K E H R K I Y T M A
672 121	GC G AC G C TCTGTGAGTGAGAACAGGTGTCACCTTGAAG S V S E N R C H L E L S R Q P

The Contract

### FIG. 1A(2)

#### CTGTGTGGCCCTGTGTGTCGGAAAGATGGAGCAAGA

AGCCGC GC TTCTC TCG TCGAGCT TG ACGAC CTTTCGCAGCCAGGAGCACCGTCCCTCCCCGGATTA

GTCGGAA ATGCGC G AAGTAG CC T CT GAGGCCCAGGGCGTCGTGCTTCCGCAGTAGTCAGTC

ACCGCG TTCTCCT C GCCTC C
GAAAACCCCGGATGGTGAGGAGCAGCAAATGTGCA
M C

TTCCAGCTTCGGAACAAGAGACCCTGGTTAGACCAA
I P A S E Q E T L V R P

C A A A A A CTTATACTATGAAAGAGGTTCTTTTTTATCTTGGCCTT Y T M K E V L F Y L G

G G
TATATTGTTCAAATGATCTTCTAGGAGATTTGTTTG
V Y C S N D L L G D L F
V

A T A G CT A G A---TCTACAGGAACTTGGTAGTAGTCAATCAGCAGGAAT
I Y R N L V V V N Q Q E
A S --

TG T C T G C CA

GTGGGAGTGATCAAAAGGACCTTGTACAAGAGCTTC
G G S D Q K D L V Q E L

L P L A P

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## FIG. 1A(3)

83

Human nt

AGCCGAGCCCGAGGGC

CATG CGCTCA G C GTGCGTACGAGCGCCCA	Mouse nt 167 Human nt
GGGCGAGC GAGACC CCCGTGAAGGAAACTGG	Mouse nt 251 Human nt
ATACCAACATGTCTGTA N T N M S V	Mouse nt  335 Human nt  8 Human a.a.  Mouse a.a.
A AGCCATTGCTTTTGAAG K P L L L K	Mouse nt Human nt Human a.a. Mouse a.a.
G AGTATATTATGACTAAA Q Y I M T K	Mouse nt 503 Human nt 64 Human a.a. Mouse a.a.
A C G T GCGTGCCAAGCTTCTCT G V P S F S	Mouse nt 587 Human nt 92 Human a.a. Mouse a.a.
T C CATCGGACTCAGGTACA S S D S G T	Mouse nt 671 Human nt 120 Human a.a. Mouse a.a.
CA AGGAAGAGAAACCTTCA Q E E K P S P	Mouse nt 755 Human nt 148 Human a.a. Mouse a.a.

# FIG. 18(1)

		$\mathbf{T}^{\prime}$	G	A	A			TG	
756	TCT	TCA	CAT	TTG	GTT	TCT	AGA	CCA	TCT
149	S	s	H	L	V	S	R	P	S
			D		I			L	
	G	G	G	С	C G	G		G	GG
840	GGT								
177	G	E	R	Q	R	K	R	H	K
				H				R	R
								A C	
924	ATA	TGT						TGT	
205						-	-	C	E
	M		S	G	G	T	S	S	S
			_		_			_	_
								C	
993									
228	V	S	E	H	S	G	D		L
								С	
	C			~		~		С	
1077									
256	S				AGC S				
230	8	11	ט	_	3	ш	3	ט די	Ц
								D	
	А	A	С			С	$\mathbf{T}$		
1161									GAA
284	G	E	S	D	${f T}$	D	S	F	E
		T					С		А
1245	AAT	_	CCC	CTT	CCA	TCA	C CAT	TGC	
12 <b>45</b> 3 <b>12</b>	AAT N	ccc	CCC P	CTT L		TCA S	C CAT H	TGC C	
		ccc							AAC
		ccc		L	P				AAC N
312	N	CCC P	P	L A	P	S	H	С	AAC N K
		CCC P ATC	P TCT	L A GAG	P AAA	s GCC	H AAA	С	AAC N K

G

# FIG. 18(2)

T C

TCA!	TCT2	AGAZ	AGGZ	AGA(	GCAI	ATTI	AGTO	SAG	CAC	SAAC	SAA
S	S	R	R	R	A S	I	S	E	T	E	E
				G				CCG			G
GAT	AGT										
-	_	_	_	_		_	_				
								_			_
	S	S							P	S	N
							E				Н
				~				_	_		
03 <i>0</i> /	m		<b></b>								
Q	ע	S	V	8	ט	Q	F,	S	V	E	F
С	G				G				С		GG
CAAC	GAAC	CTCI	CAC	SATO	AAG	ATO	ATG	AGG	rat:	אייכ	AA!
H											Ŕ
D	P	E	I	S	L	A	D	Y	W	K	C
C	2	1				C			7	C	
•	**		ш	K	Ŀ	14	π	T	2	_	ט
		_								ע	
G	3 T	G	А		Α		G		G		
							TTG	ATG		CTG	AT
	A						L				
	GATI D CAGC PCAN CAGC CAAC Q CAAC Q CAAC CAAC CAAC CAAC	GATAGTA D S CAGGATA S S CAGGATA Q D CAAGAAC Q E H GATCCTC D P CAACAC C W GTTCCAACAC S T	S S R  GATAGTATT  D S I  CAGCAGTAGC  AGCAGTAGC  S S S  CAGGATTCAC  Q D S  CAAGAACTC  Q E L  H  GATCCTGAA  D P E  CAACCACAA  GT G  CCAACACAA  S T Q	S S R R  GATAGTATTTCC D S I S CAGCAGTAGCAGT S S S S  CAGCAGTAGCAGT Q D S V  CAAGAACTCTCAC Q E L S H  GATCCTGAAATT D P E I  C A CGTTGGGCCCTTC C W A L T  G T G A CCAACACAAGCT S T Q A	S S R R R  GATAGTATTTCCCTTT D S I S L C C G AGCAGTAGCAGTGAAT S S S S E  CAGGATTCAGTTCAC Q D S V S  CAAGAACTCTCAGATC Q E L S D H  GATCCTGAAATTTCCT D P E I S  C G CAAGGACCTCTCGTC C W A L R T  G T G A CCAACACAAGCTGAAC S T Q A E	S S R R R A S  G GATAGTATTTCCCTTTCCT D S I S L S C C G C AGCAGTAGCAGTGAATCTA S S S S E S  CAGGATTCAGTTTCAGATCA Q D S V S D  C G G G CAAGAACTCTCAGATGAA Q E L S D E H  GATCCTGAAATTTCCTTAG D P E I S L  C A CGTGGGCCCTTCGTGAGA C W A L R E T  G T G A A CCAACACAAGCTGAAGAGG S T Q A E E	S S R R R A I S  G  GATAGTATTTCCCTTTCCTTTC D S I S L S F   C C G C  AGCAGTAGCAGTGAATCTACAC S S S S E S T  CAGGATTCAGTTCAGATCAGT Q D S V S D Q  C G G  CAAGAACTCTCAGATGAAGATC Q E L S D E D H  GATCCTGAAATTTCCTTAGCTC D P E I S L A  C A C  GGTTGGGCCCTTCGTGAGAATT C W A L R E N  T  G T G A A  CCAACACAAGCTGAAGAGGGCT S T Q A E E G	S S R R R A I S S  GATAGTATTTCCCTTTCCTTTGATC D S I S L S F D  C C G C A  AGCAGTAGCAGTGAATCTACAGGGA S S S S E S T G E  CAGGATTCAGTTTCAGATCAGTTTA Q D S V S D Q F  CAAGAACTCTCAGATGAAGATGATC Q E L S D E D D  H  GATCCTGAAATTTCCTTAGCTGACT D P E I S L A D  C A C CGTTGGGCCCTTCGTGAGAATTGGC C W A L R E N W  T  G T G A A G CCAACACAAGCTGAAGAGGGCTTTG S T Q A E E G F	S S R R R A I S E  S  CCG  GATAGTATTTCCCTTTCCTTTGATGAAA  D S I S L S F D E  C C G C A  AGCAGTAGCAGTGAATCTACAGGGACGC S S S S E S T G T  E  CAGGATTCAGTTTCAGATCAGTTTAGTC Q D S V S D Q F S  CAAGAACTCTCAGATGAAGATGATGAGG Q E L S D E D D E  H  GATCCTGAAATTTCCTTAGCTGACTATT D P E I S L A D Y  C A C  CGTTGGGCCCTTCGTGAGAATTGGCTTC C W A L R E N W L  T  G T G A A G  CCAACACAAGCTGAAGAGGGGCTTTGATG S T Q A E E G F D	S S R R R A I S E T  CCG  GATAGTATTTCCCTTTCCTTTGATGAAAGCC  D S I S L S F D E S  C C G C A C  AGCAGTAGCAGTGAATCTACAGGGACGCCAT S S S S E S T G T P  E  CAGGATTCAGTTTCAGATCAGTTTAGTGTAG Q D S V S D Q F S V  CAAGAAACTCTCAGATGAAGATGATGAGGTAT Q E L S D E D D E V  H  GATCCTGAAATTTCCTTAGCTGACTATTGGA D P E I S L A D Y W  CC A C A  CGTTGGGCCCTTCGTGAGAATTGGCTTCCTG C W A L R E N W L P  T  G T G A A G  CCAACACAAGCTGAAGAGGGGCTTTGATGTTC S T Q A E E G F D V	GATAGTATTTCCTTTCCTTTGATGAAAGCCTGC DSISLSFDESL  CCCGCACACACAAGCTGAAAATTCCTTAGTGAAAATTCCTTAGCTGACTATTGGAAAATCCCTGAAAATTCCTTAGCTGACTATTGGAAAATCCCTGAAAATTCCTTAGCTGACTATTGGAAAATCCCTGAAAATTCCTTAGCTGACTATTGGAAAATCCCTGAAAATTCCTTAGCTGACTATTGGAAAATCCCTGAAAATTCCTTAGCTGACTATTGGAAAATCCCTGAAAATTCCTTAGCTGACTATTGGAAAATCCCTGAAAATTCCTTAGCTGACTATTGGAAAATCCCTGAAAATTCCTTAGCTGACTATTGGAAAATCCCTGAAAATTCCTTAGCTGACTATTGGAAAATCCCTGAAAATTCCTTAGCTGACTATTGGAAAATCCCTGAAAATTTCCTTAGCTGACTATTGGAAAATCCCTGAAAATTCCTTAGCTGACTATTGGAAAATCCCTGAAAATTCCCTTAGCTGACTATTGGAAAATCCCTGAAAATCCCTGAAAAATCCCTGAAAAATATCCTTAGCTGAAAATTGGCTTCCTGAAAATCCCTTAAAAAAAA

# FIG. 18(3)

CA <b>AATTCA</b>		C C		839	Mouse <b>Human</b>	_
N S			S	176	Human	a.a.
T	~ ~		P	_, ,	Mouse	
	AGC G				Mouse	
CTGTGT	GTAATA	AGGG	AG	923	Human	nt
L C	V I	R	E	204	Human	a.a.
	E L				Mouse	a.a.
A			С		Mouse	
CCGGAT				992	Human	
P D	L D	A	G	227	Human	
Q		D			Mouse	a.a.
	G	G			Mouse	nt
GAAGTT			AC 1	1076	Human	
E V	E S	L	D	255	Human	a.a.
					Mouse	a.a.
C A	С	A			Mouse	nt
C A GTTACT	-			160	Mouse <b>Huma</b> n	
	-	CAGG		160 283		nt
GTTACT	GTGTAT	CAGG Q	CA 1		Human	nt a.a.
GTTACT	GTGTAT	CAGG Q	CA 1 A		Human Human	nt a.a. a.a.
GTTACT V T	GTGTAT V Y	CAGG Q	CA 1 A T		Human Human Mouse	<pre>nt a.a. a.a. nt</pre>
GTTACT V T	GTGTAT V Y	CAGG Q GAAA	CA 1 A T	283	Human Human Mouse Mouse	<pre>nt a.a. a.a. nt nt</pre>
GTTACT V T C ACTTCA	GTGTAT V Y TGCAAT	CAGG Q GAAA	CA 1 A T	283	Human Human Mouse Mouse Human	<pre>nt a.a. a.a. nt nt a.a.</pre>
GTTACT V T C ACTTCA	GTGTAT V Y TGCAAT	CAGG Q GAAA E	CA 1 A T	283	Human Human Mouse Mouse Human Human	nt a.a. a.a. nt nt a.a. a.a.
GTTACT V T  C ACTTCA T S	GTGTAT V Y  TGCAAT C N	CAGG Q GAAA E	CA 1 A T TG 1 M	283	Human Human Mouse Mouse Human Human Mouse	nt a.a. a.a. nt a.a. nt
GTTACT V T  C ACTTCA T S	GTGTAT V Y  TGCAAT C N	CAGG Q GAAA E	CA 1 A T TG 1 M	283 1244 311	Human Human Mouse Mouse Human Human Mouse	<pre>nt a.a. a.a. nt nt a.a. a.a. nt nt</pre>
GTTACT V T  C ACTTCA T S  G AAAGGG	GTGTAT V Y  TGCAAT C N  AAAGAT	CAGG Q GAAA E AAAG K	CA 1 A T TG 1 M T GG 1	283 1244 311	Human Human Mouse Human Human Mouse Mouse	nt a.a.  nt nt a.a.  nt nt a.a.
GTTACT V T  C ACTTCA T S  G AAAGGG K G	GTGTAT V Y TGCAAT C N AAAGAT K D	CAGG Q GAAA E AAAG K	CA 1 A T TG 1 M T GG 1	283 1244 311	Human Human Mouse Human Human Mouse Mouse Human Human Human	nt a.a.  nt a.a.  nt a.a.  nt nt a.a.
GTTACT V T  C ACTTCA T S  G AAAGGG K G	GTGTAT V Y TGCAAT C N AAAGAT K D	CAGG Q GAAA E AAAG K	CA 1 A T TG 1 M T GG 1 G V	283 1244 311 1228 339	Human Human Mouse Human Human Mouse Mouse Human Human Human Human	nt a.a. nt nt a.a. nt nt a.a. nt
GTTACT V T  C ACTTCA T S  G AAAGGG K G  G C TGTAAA	GTGTAT V Y TGCAAT C N AAAGAT K D GCTG	CAGG Q GAAA E AAAG K	CA A T TG M T GG V A TG 1	283 1244 311 1228 339	Human Human Mouse Human Human Mouse Mouse Human Human Human Human Human	nt a.a.  nt nt a.a.  nt nt a.a.  nt nt nt nt
GTTACT V T  C ACTTCA T S  G AAAGGG K G	GTGTAT V Y TGCAAT C N AAAGAT K D	CAGG Q GAAA E AAAG K	CA 1 A T TG 1 M T GG 1 G V	283 1244 311 1228 339	Human Human Mouse Human Human Mouse Mouse Human Human Human Human	nt a.a.  nt nt a.a.  nt nt a.a.  nt nt a.a.

# FIG. IC(1)

			GI	A		C		C		G
1413	AAT	GAT	TCC	AGA			TGT	GTT	GAG	GAA
368	N	D	S	R	E	S	C	V	E	E
			Α	K		P		Α		
	С	A		G		С	C			G
1494	TCT	CAG	CCA	TCA	ACT	TCT	AGT	AGC	ATT	TTA
395	S	Q	P	S	T	S	S	S	I	I
										V
	С									G
1578	GAA									
423	E	E	S	V	E	S	S			L
	D							F	S	
	m.	~		<b>a</b>	~		~			
1.660	T GTC	_		G						
1662 451	V		G							
451	V	п	G	K	T	G	л	L	M	<b>A</b> S
										3
		G		С						G
1746	AGA	CAA	CCA	ATT	CAA	ATG	ATT	GTG	CTA	
479		Q	P	I	0	M	I	V	L	${f T}$
		_			-					S
1830	TAA									
1914	TTA									
1998	ACT									
2082	ATG'									
2166	CTC									
2250	TAA									
2334	CTC	GGC	CTC	CCA	AAG	TGC	TGG	GAT	TAC	AGG

## FIG. IC(2)

											C
											TCAC
N	1 -	D	D	K	1	T	Q	A	<b>D</b>	δ	Ь
D	S	E	E		Α	E		.1.	Р	T	
					AGC				G		A
TA	TAG	CAG	CCA	AGAA	GAT	GTG	AAA	GAG	TTT	GAA	AGGG
	S										
			~	•	S				L	-	K
		i	С	A			С	C	G	G	G
AA	TGC	CAT	TGA	ACCI	TGT	GTG	ATT	TGT	CAA	GGT	CGAC
N	I A	I	E	P	C	V	I	C	Q	G	R
	${f T}$	C	G					A	,	A	С
TG	CTT	TAC	ATG	rgca	AAG	AAG	CTA	AAG	AAA	AGG	AATA
C	F	T	C	A	K	K	L	K	K	R	N

C AA C CTCA A A T

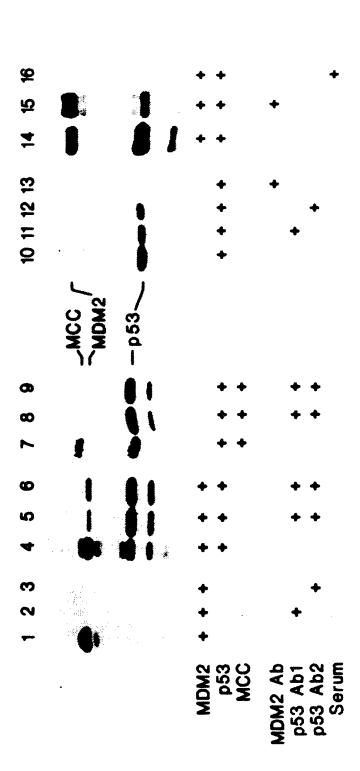
TATTTCCCCTAGTTGACCTG---TCTATAAGAGAATT
Y F P
N

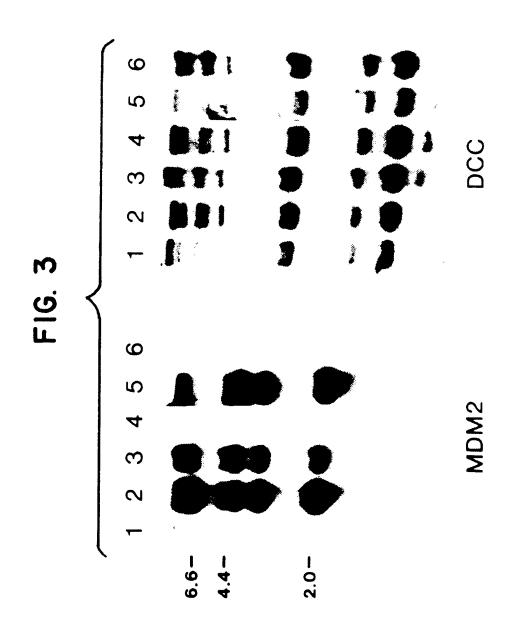
Sulface (Liberal)

# FIG. 1C(3)

G G C  AAGAAAGTGAAGACTAT  Q E S E D Y  D	1493 3 <b>94</b>	Mouse Human Human Mouse	nt a.a.
G G GC  AAGAAACCCAAGACAAA E E T Q D K H	1577 422	Mouse Human Human Mouse	
C CTAAAAATGGTTGCATT P K N G C I	1661 450	Mouse Human Human Mouse	nt a.a.
G C AGCCCTGCCCAGTATGT K P C P V C	1745 478	Mouse Human Human Mouse	nt nt a.a. a.a.
T * ATATATTTCTAACTATA	1829 491	Mouse Human Human Mouse	nt
ACATAGATTTCTTCTCT GCTCATCCTTTACACCA ATGTATATGACATTTAA TCTTGGCTCACTGCAAG CTGCCACCACACCTGGC CCTCGTGATCCGCCCAC	1913 1997 2081 2165 2249 2333 2372	Human Human Human Human Human Human	nt nt nt nt nt







1 2 3 4
FIG. 4A
-7.5
-4.4

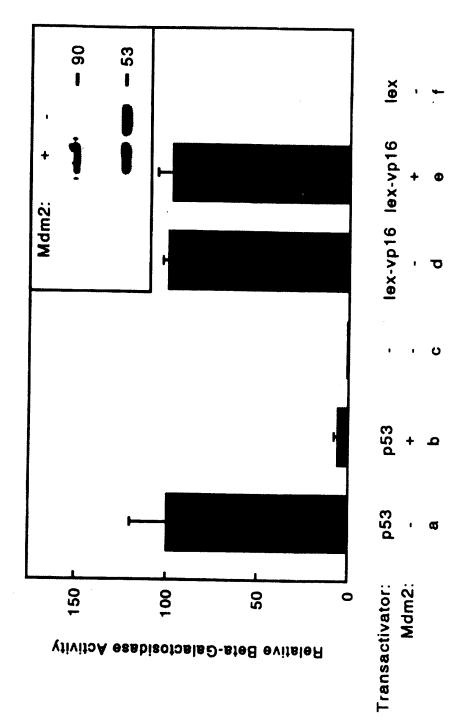
1 2 3 4 -200 FIG. 4B -97

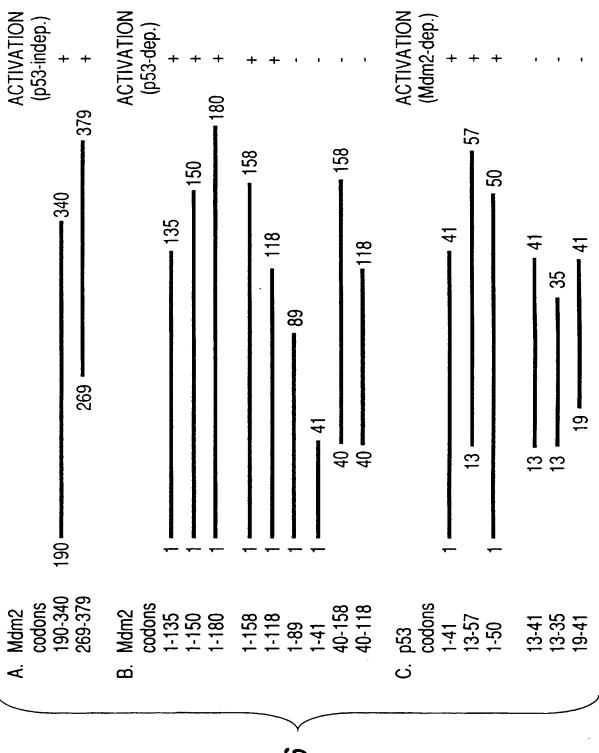
1 2 3 4 5

FIG. 4C

- 200
- 97

F16. 5





-1G. 6

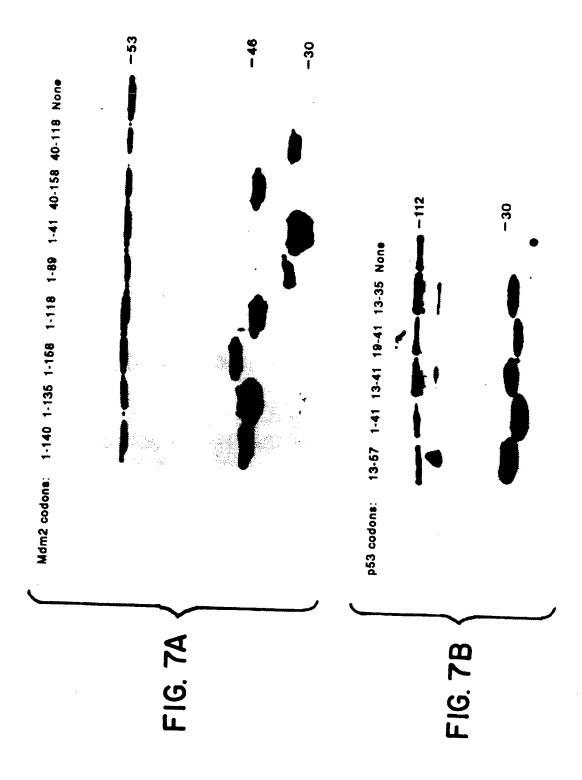
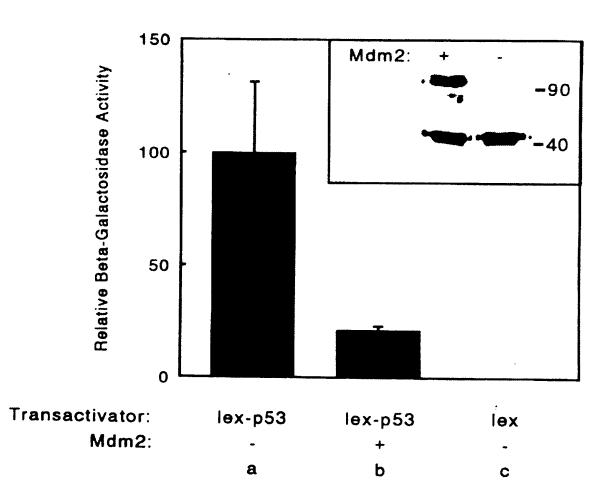
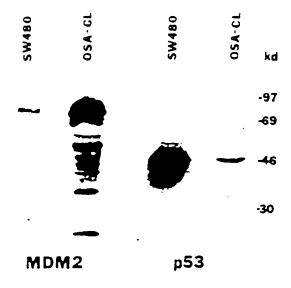


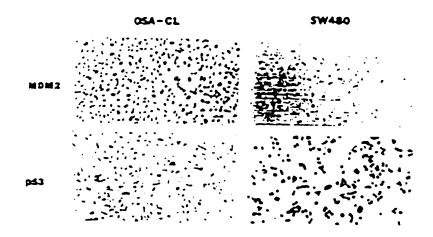
FIG. 8



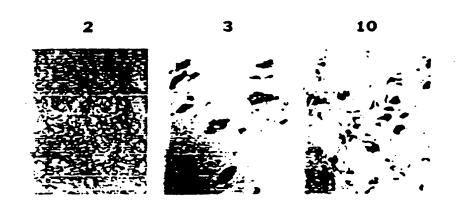
## FIGURE 9



## FIGURE 10



## FIGURE 11



PCT/US 93/03199

			Isterational Application N	PCT/US 93/03199
I. CLASSIF	CATTON OF SUBJ	CT MATTER (If several cleanifi		
		Classification (IPC) or to both Na		
Int.Cl.	5 C12Q1/68	G01N33/57	74; C07H21/00	
L PIELDS	SEARCHED			
		Minimum	Documentation Searched	
Classification	os System		Classification Symbols	
Int.Cl.	5	C12Q		
			d other than Minimum Documentanon iments are included in the Fields Searched <sup>8</sup>	
III. DOCUM		D TO BE RELEVANT		
Category *	Citation of Do	cument, it with indication, where a	ppropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No.13
4	EMBO JOU vol. 10,	RNAL. no. 6, 1991, EYNS	HAM, OXFORD GB	1-55
	pages 15 FAKHARZA associat gene tha cell lin cited in	65 - 1569 DEH ET AL. 'Tumori ed with enhanced e t is amplified in	ginic potential xpression of a	
	SNYDER L TRANSFOR COMPLEX	HEM 263 (32). 1988 C ET AL. 'A GENE / MED MOUSE CELL LINI TRANSCRIPTIONAL PRO A NUCLEAR PROTEIN.	AMPLIFIED IN A E UNDERGOES DCESSING AND	-
			-/-	-
"A" decan creation filing "C" decan citation cit	references to be of partical or document but publis grate ment which may throw its cited to establish to on or other special run ment referring to an or ment published prior to than the priority date.	rai state of the art which is not ar relevance hed on or after the international doubts on priority claim(s) or he publication date of another son (as specified) rai disclosure, see, exhibition or to the international filling date but	"T" inter document published after or priority date and not in one cited to understand the practitiventies.  "X" document of particular relevanceanes be considered novel or inventive step.  "Y" document of particular relevanceanes to combined with sements is combined with sements, such combination being in the art.  "A" document member of the same	idict with the application but ple or theory underlying the form the claimed invention connect be considered to now the claimed invention we an inventive step when the he or more other such docu- g obvious to a person skilled
V. CERTER	CATION  Steel Completion of th	e International Security	Personal Marilla and other tra	
	17 SEPTEMBI		Date of Mailing of this interm	0 1. 10. 93
Attacheen) (	EUROPEA	N PATENT OFFICE	Signature of Authorized Office MOLINA GALAN	

	International Application N  II. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)							
Category *	Citation of Document, with indication, where appropriate, or the resevant passages	ABOVED TO CIME ITO						
<b>A</b>	CELL GROWTH & DIFFERENTIATION vol. 1, 1990, pages 571 - 580 HINDS ET AL. 'Mutant p53 DNA clones from human colon carcinomas cooperate with ras in transforming primary rat cells: a comparison of the "hot spot" mutant phenotypes' cited in the application							
<b>A</b>	EP,A,0 341 904 (TEMPLE UNIVERSITY) 15 November 1989 see abstract	30-33						
A	US,A,4 968 603 (SLAMON ET AL.) 6 November 1990	-						
P,X	NATURE vol. 358, 2 July 1992, LONDON GB pages 80 - 83 OLINER ET AL. 'Amplification of a gene encoding a p53 associated protein in human sarcomas' see the whole document	1,18,20						
P,X	CELL 69 (7). 1992. 1237-1245. MOMAND J ET AL. 'THE MDM -2 ONCOGENE PRODUCT FORMS A COMPLEX WITH THE'	20						

## ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9303199 SA 73548

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.

The members are as contained in the European Patent Office EDP file on

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17/09/93

Patent document cited in search report	Publication date		t family aber(s)	Publication date
EP-A-0341904	15-11-89	JP-A-	2013400	17-01-90
US-A-4968603	06-11-90	None		



## WORLD INTELLECTUAL PROPERTY ORGANIZATION International Bureau



1)

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C07H 21/00		13) International Publication Date: 14 October 1993 (14.10.9
(21) International Application Number: PCT/US (22) International Filing Date: 7 April 1993		& Beckett, 1001 G Street, N.W., 11th Floor, Washingto
(30) Priority data: 867,840 7 April 1992 (07.04.92) 903,103 23 June 1992 (23.06.92)		
<ul> <li>(71) Applicant: THE JOHNS HOPKINS UNIVERS US]; 720 Rutland Avenue, Baltimore, MD 212</li> <li>(72) Inventors: BURRELL, Marilee; 198 Hampshi Cambridge, MA 02139 (US). HILL, David, E.; STreet, Arlington, MA 02174 (US). KINZLER, W.; 1348 Halstead Road, Baltimore, MA 21. VOGELSTEIN, Bert; 3700 Breton Way, Baltim 21208 (US).</li> </ul>	ire Stre 85 Ric Kenne 234 (U	With international search report.  Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.  (88) Date of publication of the international search report.

(54) Title: AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

#### (57) Abstract

A human gene has been discovered which is genetically altered in human tumor cells. The genetic alteration is gene amplification and leads to a corresponding increase in gene products. Detecting that the gene, designated hMDM2, has become amplified or detecting increased expression of gene products is diagnostic of tumorigenesis. Human MDM2 protein binds to human p53 and allows the cell to escape from p53-regulated growth.

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## AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS

This application is a continuation-in-part of United States Serial No. 07/903,103, filed June 23, 1992, which is a continuation-in-part of United States Serial No. 07/867,840, filed April 7, 1992, now abandoned.

This invention was made with support from the U.S. Government, including NIH grants CA-57345, CA-43460, CA-02243 and CA-35494. Accordingly, the Government retains certain rights in the invention.

### FIELD OF THE INVENTION

The invention relates to the area of cancer diagnostics and therapeutics. More particularly, the invention relates to the detection of a gene which is amplified in certain human tumors.

### BACKGROUND OF THE INVENTION

According to the Knudson model for tumorigenesis (Cancer Research, 1985, vol. 45, p. 1482), there are tumor suppressor genes in all normal cells which, when they become non-functional due to mutation, cause neoplastic development. Evidence for this model has been found in cases of retinoblastoma and colorectal tumors. The implicated suppressor genes in these tumors, RB and p53 respectively, were found to be deleted or altered in many of the tumors studied.

The p53 gene product, therefore, appears to be a member of a group of proteins which regulate normal cellular proliferation and suppression of cellular transformation. Mutations in the p53 gene have been linked to tumorigenesis, suggesting that alterations

in p53 protein function are involved in cellular transformation. The inactivation of the p53 gene has been implicated in the genesis or progression of a wide variety of carcinomas (Nigro et al., 1989, Nature 342:705-708), including human colorectal carcinoma (Baker et al., 1989, Science 244:217-221), human lung cancer (Takahashi et al., 1989, Science 246:491-494; Iggo et al., 1990, Lancet 335:675-679), chronic myelogenous leukemia (Kelman et al., 1989, Proc. Natl. Acad. Sci. USA 86:6783-6787) and osteogenic sarcomas (Masuda et al., 1987, Proc. Natl. Acad. Sci. USA 84:7716-7719).

While there exists an enormous body of evidence linking p53 gene mutations to human tumorigenesis (Hollstein et al., 1991, Science 253:49-53) little is known about cellular regulators and mediators of p53 function.

Hinds et al. (Cell Growth & Differentiation, 1:571-580, 1990), found that p53 cDNA clones, containing a point mutation at amino acid residue 143, 175, 273 or 281, cooperated with the activated ras oncogene to transform primary rat embryo fibroblasts in culture. These mutant p53 genes are representative of the majority of mutations found in human cancer. Hollstein et al., 1991, Science 253:49-53. The transformed fibroblasts were found to produce elevated levels of human p53 protein having extended half-lives (1.5 to 7 hours) as compared to the normal (wild-type) p53 protein (20 to 30 minutes).

Mutant p53 proteins with mutations at residue 143 or 175 form an oligomeric protein complex with the cellular heat shock protein hsc70. While residue 273 or 281 mutants do not detectably bind hsc70, and are poorer at producing transformed foci than the 175 mutant, complex formation between mutant p53 and hsc70 is not required for p53-mediated transformation. Complex formation does, however, appear to facilitate this function. All cell lines transformed with the mutant p53 genes are tumorigenic in a thymic (nude) mice. In contrast, the wild-type human p53 gene does not possess transforming activity in cooperation with ras. Tuck and Crawford, 1989, Oncogene Res. 4:81-96.

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Hinds et al., supra also expressed human p53 protein in transformed rat cells. When the expressed human p53 was immunoprecipitated with two p53 specific antibodies directed against distinct epitopes of p53, an unidentified  $M_r$  90,000 protein was coimmunoprecipitated. This suggested that the rat  $M_r$  90,000 protein is in a complex with the human p53 protein in the transformed rat cell line.

As mentioned above, levels of p53 protein are often higher in transformed cells than normal cells. This is due to mutations which increase its metabolic stability (Oven et al., 1981, Mol. Cell. Biol. 1:101-110; Reich et al. (1983), Mol. Cell. Biol. 3:2143-2150). The stabilization of p53 has been associated with complex formation between p53 and viral or cellular proteins. (Linzer and Levine, 1979, Cell 17:43-52; Crawford et al., 1981, Proc. Natl. Acad. Sci. USA 78:41-45; Dippold et al., 1981, Proc. Natl. Acad. Sci. USA 78:1695-1699; Lane and Crawford, 1979, Nature (Lond.) 278:261-263; Hinds et al., 1987, Mol. Cell. Biol. 7:2863-2869; Finlay et al., 1988, Mol. Cell. Biol. 8:531-539; Sarnow et al., 1982, Cell. 28:387-394; Gronostajski et al., 1984, Mol. Cell. Biol. 4:442-448; Pinhasi-Kimhi et al., 1986, Nature (Lond.) 320:182-185; Ruscetti and Scolnick, 1983, J. Virol. 46:1022-1026; Pinhasi and Oren, 1984, Mol. Cell. Biol. 4:2180-2186; and Sturzbecher et al., 1987, Oncogene 1:201-211.) For example, p53 protein has been observed to form oligomeric protein complexes with the SV40 large T antigen, the adenovirus type 5 E1B-M, 55,000 protein, and the human papilloma virus type 16 or 18 E6 product. Linzer and Levine, 1979. Cell 17:43-52; Lane and Crawford, 1979, Nature, 278:261-263; Sarnow et al., 1982, Cell 28:387-394; Werness et al., 1990, Science, 248:76-79. Similarly, complexes have been observed of p105RB (the product of the retinoblastoma susceptibility gene) with T antigen (DeCaprio et al., 1988, Cell 54:275-283), the adenovirus EIA protein (Whyte et al., 1988, Nature 334:124-129) and the E7 protein of human papilloma virus 16 or 18 (Münger et al., 1989, EMBO J. 8:4099-4105). It has been suggested that interactions between these viral proteins and p105RB inactivate a growth-suppressive function of p105<sup>RB</sup>, mimicking deletions and mutations commonly found in the RB gene in tumor cells. In a similar fashion, oligomeric protein complex

formation between these viral proteins and p53 may eliminate or alter the function of p53. Finlay et al., 1989, Cell 57:1083-1093.

Fakharzadeh et al. (EMBO J. 10:1565-1569, 1991) analyzed amplified DNA sequences present in a tumorigenic mouse cell line (i.e., 3T3DM, a spontaneously transformed derivative of mouse Balb/c cells). Studies were conducted to determine whether any of the amplified genes induced tumorigenicity following introduction of the amplified genes into a nontransformed recipient cell (e.g., mouse NIH3T3 or Rat2 cells). The resulting cell lines were tested for tumorigenicity in nude mice. A gene, designated MDM2, which is amplified more than 50-fold in 3T3DM cells, induced tumorigenicity when overexpressed in NIH3T3 and Rat 2 cells. From the nucleotide and predicted amino acid sequence of mouse MDM2 (mMDM2), Fakharzadeh speculated that this gene encodes a potential DNA binding protein that functions in the modulation of expression of other genes and, when present in excess, interferes with normal constraints on cell growth.

#### SUMMARY OF THE INVENTION

It is an object of the invention to provide a method for diagnosing a neoplastic tissue, such as sarcoma, in a human.

It is another object of the invention to provide a cDNA molecule encoding the sequence of human MDM2.

Yet another object of the invention is to provide a preparation of human MDM2 protein which is substantially free of other human cellular proteins.

Still another object of the invention is to provide DNA probes capable of hybridizing with human MDM2 genes or mRNA molecules.

Another object of the invention is to provide antibodies immunoreactive with human MDM2 protein.

Still another object of the invention is to provide kits for detecting amplification or elevated expression of human MDM2.

Yet another object of the invention is to provide methods for identifying compounds which interfere with the binding of human MDM2 to human p53.

A further object of the invention is to provide a method of treating a neoplastic human cell.

Yet another object of the invention is to provide methods for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification.

Still another object of the invention is to provide polypeptides which interfere with the binding of human MDM2 to human p53.

A further object of the invention is to provide a method for growing host cells containing a p53 expression vector.

It has now been discovered that hMDM2, a heretofore unknown human gene, plays a role in human cancer. The hMDM2 gene has been cloned and the recombinant derived hMDM2 protein shown to bind to human p53 in vitro. hMDM2 has been found to be amplified in some neoplastic cells and the expression of hMDM2-encoded products has been found to be correspondingly elevated in tumors with amplification of this gene. The elevated levels of MDM2 appear to sequester p53 and allow the cell to escape from p53-regulated growth.

### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-C shows the cDNA sequence of human MDM2. In this figure, human and mouse nucleotide and amino acid sequences are compared, the mouse sequence being shown only where it differs from the corresponding human sequence.

Figure 2 shows that hMDM2 binds to p53.

Figure 3 illustrates the amplification of the hMDM2 gene in sarcomas.

Figure 4A-C illustrates hMDM2 expression.

Figure 5 shows the inhibition of p53-mediated transactivation by MDM2. Yeast were stably transfected with expression plasmids encoding p53, lex-VP16, MDM2 or the appropriate vector-only controls, as indicated. p53-responsive (bars a-c) or lexA-responsive (bars d-f)  $\beta$ -galactosidase reporter plasmids were used to assess the response.

Inset: Western blot analysis demonstrating MDM2 (90 kD) and p53 (53 kD) expression in representative yeast strains. The strain indicated by a plus was transfected with expression vector encoding full length MDM2 and p53, while the strain indicated by a minus was transfected only with the p53 expression vector.

Figure 6 shows the determination of MDM2 and p53 domains of interaction. Fig. 5A and Fig. 5B. Random fragments of MDM2 were fused to sequences encoding the lexA DNA binding domain and the resultant clones transfected into yeast carrying pRS314SN (p53 expression vector) and pJK103 (lexA-responsive  $\beta$ -galactosidase reporter). Yeast clones expressing  $\beta$ -galactosidase were identified by their blue color, and the MDM2 sequences in the lexA fusion vector were determined.  $\beta$ -galactosidase activity was observed independent of p53 expression in A, but was dependent on p53 expression in B. The bottom 6 clones in B were generated by genetic engineering. Fig. 6C. Random fragments of p53 were fused to the sequence encoding the B42 acidic activation domain and a hemagglutinin epitope tag; the resultant clones were transfected into yeast carrying lexA-MDM2 (lexA DNA binding domain fused to full length MDM2) and pJK103. Yeast clones were identified as above, and all were found to be MDM2-dependent. The bottom three clones were generated by genetic engineering.

Figure 7 shows protein expression from the yeast strains described in Figure 6. Western blot analysis was performed as described (Oliner, J.D., et al., Nature 358:80-83 (1992)), using 20 μg of protein per lane. The MDM2 and p53 codons contained in the fusion vectors are shown at the top of A and B, respectively. Fig. 7A. Upper panel probed with p53 Ab2 detecting p53; lower panel probed with anti-lexA polyclonal antibodies (lex Ab) detecting MDM2 fusion proteins of 30-50 kD. Fig. 7B. Upper panel probed with Lex Ab detecting the lexA-full length MDM2 fusion protein of 112 kD; lower panel probed with HA Ab (a monoclonal antibody directed against the hemagglutinin epitope tag, Berkeley Antibody) detecting p53 fusion proteins of approximately 25-30 kD.

Figure 8 shows the inhibition of the p53 activation domain by MDM2. Yeast were transfected with expression vectors encoding a lexA-p53 (p53 codons 1-73) fusion (bars a and b) or lexA alone (bar c). Strain b also expressed full length MDM2, and all strains contained the lexA-responsive  $\beta$ -galactosidase reporter plasmid. Inset: Upper panel probed with MDM2 polyclonal antibodies detecting full length MDM2 (90 kD); lower panel probed with lex Ab detecting the lex-p53 fusion protein of 40 kD.

Figure 9 shows a Western blot analysis using monoclonal antibodies to MDM2 or p53. Fifty  $\mu$ g of total cellular proteins from OsA-CL or SW480 cells were used for Western blot analysis. The position of molecular weight markers, in kd, is given on the right.

Figure 10 demonstrates immunocytochemical analysis of OsA-CL and SW480 cells grown in vitro. Monoclonal antibody IF-2, specific for MDM2, and mAb 1801, specific for p53, were used. The exclusively nuclear localization of both proteins is evident, as is the higher expression of MDM2 protein in OsA-CL cells than in SW480 cells, the reverse of the pattern observed for p53.

Figure 11 demonstrates MDM2 expression in primary soft tissue sarcomas. Cryostat sections of human sarcomas were incubated with the IF-2 antibody specific for MDM2. Tumors #3 and #10 showed nuclear expression of MDM2, while tumor #2 showed no staining.

## DETAILED DESCRIPTION OF THE INVENTION

It is a discovery of the present invention that a gene exists which is amplified in some human tumors. The amplification of this gene, designated MDM2, is diagnostic of neoplasia or the potential therefor. Detecting the elevated expression of human MDM2-encoded products is also diagnostic of neoplasia or the potential for neoplastic transformation. Over a third of the sarcomas surveyed, including the most common bone and soft tissue forms, were found to have amplified hMDM2 sequences. Expression of hMDM2 was found to be correspondingly elevated in tumors with the gene amplification.

Other genetic alterations leading to elevated hMDM2 expression may be involved in tumorigenesis also, such as mutations in regulatory regions of the gene. Elevated expression of hMDM2 may also be involved in tumors other than sarcomas including but not limited to those in which p53 inactivation has been implicated. These include colorectal carcinoma, lung cancer and chronic myelogenous leukemia.

According to one embodiment of the invention, a method of diagnosing a neoplastic tissue in a human is provided. Tissue or body fluid is isolated from a human, and the copy number of human MDM2 genes is determined. Alternatively, expression levels of human MDM2 gene products can be determined. These include protein and mRNA.

Body fluids which may be tested include urine, serum, blood, feces, saliva, and the like. Tissues suspected of being neoplastic are desirably separated from normal appearing tissue for analysis. This can be done by paraffin or cryostat sectioning or flow cytometry, as is known in the art. Failure to separate neoplastic from non-neoplastic cells can confound the analysis. Adjacent non-neoplastic tissue or any normal tissue can be used to determine a base-line level of expression or copy number, against which the amount of hMDM2 gene or gene products can be compared.

The human MDM2 gene is considered to be amplified if the cell contains more than the normal copy number (2) of this gene per genome. The various techniques for detecting gene amplification are well known in the art. Gene amplification can be determined, for example, by Southern blot analysis, as described in Example 4, wherein cellular DNA from a human tissue is digested, separated, and transferred to a filter where it is hybridized with a probe containing complementary nucleic acids. Alternatively, quantitative polymerase chain reaction (PCR) employing primers can be used to determine gene amplification. Appropriate primers will bind to sequences that bracket human MDM2 coding sequences. Other techniques for determining gene copy number as are known in the art can be used without limitation.

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The gene product which is measured may be either mRNA or protein. The term elevated expression means an increase in mRNA production or protein production over that which is normally produced by non-cancerous cells. Although amplification has been observed in human sarcomas, other genetic alterations leading to elevated expression of MDM2 may be present in these or other tumors. Other tumors include those of lung, breast, brain, colorectal, bladder, prostate, liver, skin, and stomach. These, too, are contemplated by the present invention. Non-cancerous cells for use in determining baseline expression levels can be obtained from cells surrounding a tumor, from other humans or from human cell lines. Any increase can have diagnostic value, but generally the mRNA or protein expression will be elevated at least about 3-fold, 5-fold, and in some cases up to about 100-fold over that found in non-cancerous cells. The particular technique employed for detecting mRNA or protein is not critical to the practice of the invention. Increased production of mRNA or protein may be detected, for example, using the techniques of Northern blot analysis or Western blot analysis, respectively, as described in Example 4 or other known techniques such as ELISA, immunoprecipitation, RIA and the like. These techniques are also well known to the skilled artisan.

According to another embodiment of the invention, nucleic acid probes or primers for the determining of human MDM2 gene amplification or elevated expression of mRNA are provided. The probe may comprise ribo- or deoxyribonucleic acids and may contain the entire human MDM2 coding sequence, a sequence complementary thereto, or fragments thereof. A probe may contain, for example, nucleotides 1-949, or 1-2372 as shown in Figure 1. Generally, probes or primers will contain at least about 14 contiguous nucleotides of the human sequence but may desirably contain about 40, 50 or 100 nucleotides. Probes are typically labelled with a fluorescent tag, a radioisotope, or the like to render them easily detectable. Preferably the probes will hybridize under stringent hybridization conditions. Under such conditions they will not hybridize to mouse MDM2. The probes of the invention are complementary to the human MDM2 gene. This means that they share 100% identity with the human sequence.

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hMDM2 protein can be produced, according to the invention, substantially free of other human proteins. Provided with the DNA sequence, those of skill in the art can express the cDNA in a non-human cell. Lysates of such cells provide proteins substantially free of other human proteins. The lysates can be further purified, for example, by immunoprecipitation, co-precipitation with p53, or by affinity chromatography.

The antibodies of the invention are specifically reactive with hMDM2 protein. Preferably, they do not cross-react with MDM2 from other species. They can be polyclonal or monoclonal, and can be raised against native hMDM2 or a hMDM2 fusion protein or synthetic peptide. The antibodies are specifically immunoreactive with hMDM2 epitopes which are not present on other human proteins. Some antibodies are reactive with epitopes unique to human MDM2 and not present on the mouse homolog. The antibodies are useful in conventional analyses, such as Western blot analysis, ELISA, immunohistochemistry, and other immunological assays for the detection of proteins. Techniques for raising and purifying polyclonal antibodies are well known in the art, as are techniques for preparing monoclonal antibodies. Antibody binding can be determined by methods known in the art, such as use of an enzyme-labelled secondary antibody, staphylococcal protein A, and the like. Certain monoclonal antibodies of the invention have been deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852. These include IF2, and ED9, which have been granted accession nos. HB 11290, and HB 11291, respectively.

According to another embodiment of the invention, interference with the expression of MDM2 provides a therapeutic modality. The method can be applied in vivo, in vitro, or ex vivo. For example, expression may be down-regulated by administering triple-strand forming or antisense oligonucleotides which bind to the hMDM2 gene or mRNA, respectively, and prevent transcription or translation. The oligonucleotides may interact with unprocessed pre-mRNA or processed mRNA. Small molecules and peptides which specifically inhibit MDM2 expression can also be used.

Similarly, such molecules which inhibit the binding of MDM2 to p53 would be therapeutic by alleviating the sequestration of p53.

Such inhibitory molecules can be identified by screening for interference of the hMDM2/p53 interaction where one of the binding partners is bound to a solid support and the other partner is labeled. Antibodies specific for epitopes on hMDM2 or p53 which are involved in the binding interaction will interfere with such binding. Solid supports which may be used include any polymers which are known to bind proteins. The support may be in the form of a filter, column packing matrix, beads, and the like. Labeling of proteins can be accomplished according to any technique known in the art. Radiolabels, enzymatic labels, and fluorescent labels can be used advantageously. Alternatively, both hMDM2 and p53 may be in solution and bound molecules separated from unbound subsequently. Any separation technique known in the art may be employed, including immunoprecipitation or immunoaffinity separation with an antibody specific for the unlabeled binding partner.

It has been found that amino acid residues 13-41 of p53 (See SEQ ID NO:1) are necessary for the interaction of MDM-2 and p53. However, additional residues on either the amino or carboxy terminal side of the peptide appear also to be required. Nine to 13 additional p53 residues are sufficient to achieve MDM2 binding, although less may be necessary. Since cells which overexpress MDM2 escape from p53-regulated growth control in sarcomas, the use of p53-derived peptides to bind to excess MDM2 leads to reestablishment of p53-regulated growth control.

Suitable p53-derived peptides for administration are those which are circular, linear, or derivitized to achieve better penetration of membranes, for example. Other organic compounds which are modelled to achieve the same three dimensional structure as the peptide of the invention can also be used.

DNA encoding the MDM2-binding, p53-derived peptide, or multiple copies thereof, may also be administered to tumor cells as a mode of administering the peptide. The DNA will typically be in an expression construct, such as a retrovirus, DNA virus,

or plasmid vector, which has the DNA elements necessary for expression properly positioned to achieve expression of the MDM2-binding peptide. The DNA can be administered, *inter alia* encapsulated in liposomes, or in any other form known to the art to achieve efficient uptake by cells. As in the direct administration of peptide, the goal is to alleviate the sequestration of p53 by MDM2.

A cDNA molecule containing the coding sequence of hMDM2 can be used to produce probes and primers. In addition, it can be expressed in cultured cells, such as *E. coli*, to yield preparations of hMDM2 protein substantially free of other human proteins. The proteins produced can be purified, for example, with immunoaffinity techniques using the antibodies described above.

Kits are provided which contain the necessary reagents for determining gene copy number, such as probes or primers specific for the hMDM2 gene, as well as written instructions. The instructions can provide calibration curves to compare with the determined values. Kits are also provided to determine elevated expression of mRNA (i.e., containing probes) or hMDM2 protein (i.e., containing antibodies). Instructions will allow the tester to determine whether the expression levels are elevated. Reaction vessels and auxiliary reagents such as chromogens, buffers, enzymes, etc. may also be included in the kits.

The human !MDM2 gene has now been identified and cloned. Recombinant derived hMDM2 has been shown to bind to human p53. Moreover, it has been found that hMDM2 is amplified in some sarcomas. The amplification leads to a corresponding increase in MDM2 gene products. Such amplification is associated with the process of tumorigenesis. This discovery allows specific assays to be performed to assess the neoplastic or potential neoplastic status of a particular tissue.

The following examples are provided to exemplify various aspects of the invention and are not intended to limit the scope of the invention.

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#### **EXAMPLES**

#### Example 1

To obtain human cDNA clones, a cDNA library was screened with a murine MDM2 (mMDM2) cDNA probe. A cDNA library was prepared by using polyadenylated RNA isolated from the human colonic carcinoma cell line CaCo-2 as a template for the production of random hexamer primed double stranded cDNA. Gubler and Hoffmann, 1983, Gene 25:263-268. The cDNA was ligated to adaptors and then to the lambda YES phage vector, packaged, and plated as described by Elledge et al. (Proc. Natl. Acad. Sci. USA, 88:1731-1735, 1991). The library was screened initially with a P-labelled (Kinzler, K.W., et al., Nucl. Acids Res. 17:3645-3653 (1989), Feinberg and Vogelstein, 1983, Anal. Biochem. 132.6-13) mMDM2 cDNA probe (nucleotides 259 to 1508 (Fakharzadeh et al., 1991, EMBO J. 10:1565-1569)) and then rescreened with an hMDM2 cDNA clone containing nucleotides 40 to 702.

Twelve clones were obtained, and one of the clones was used to obtain thirteen additional clones by re-screening the same library. In total, twenty-five clones were obtained, partially or totally sequenced, and mapped. Sequence analysis of the twenty-five clones revealed several cDNA forms indicative of alternative splicing. The sequence shown in Figure 1 is representative of the most abundant class and was assembled from three clones: c14-2 (nucleotides 1-949), c89 (nucleotides 467-1737), and c33 (nucleotides 390-2372). The 3' end of the untranslated region has not yet been cloned in mouse or human. The 5' end is likely to be at or near nucleotide 1. There was an open reading frame extending from the 5' end of the human cDNA sequence to nucleotide 1784. Although the signal for translation initiation could not be unambiguously defined, the ATG at nucleotide 312 was considered the most likely position for several reasons. First, the sequence similarity between hMDM2 and mMDM2 fell off dramatically upstream of nucleotide 312. This lack of conservation in an otherwise highly conserved protein suggested that the sequences upstream of the divergence may not code for protein. Second, an anchored polymerase chain reaction (PCR) approach was employed in an

effort to acquire additional upstream cDNA sequence. Ochman et al., 1985, In: PCR Technology: Principles and Applications for DNA Amplification (Erlich, ed.) pp. 105-111 (Stockton, New York). The 5' ends of the PCR derived clones were very similar (within 3 bp) to the 5' ends of clones obtained from the cDNA library, suggesting that the 5' end of the hMDM2 sequence shown in Figure 1 may represent the 5' end of the transcript. Third, in vitro translation of the sequence shown in Figure 1, beginning with the methionine encoded by the nucleotide 312 ATG, generated a protein similar in size to that observed in human cells.

In Figure 1, hMDM2 cDNA sequence, hMDM2 and mMDM2 nucleotide and amino acid sequences are compared. The mouse sequence is only shown where it differs from the corresponding human sequence. Asterisks mark the 5' and 3' boundaries of the previously published mMDM2 cDNA. Fakharzadeh et al., 1991, EMBO J. 10:1565-1569. Dashes indicate insertions. The mouse and human amino acid sequences are compared from the putative translation start site at nucleotide 312 through the conserved stop codon at nucleotide 1784.

Comparison of the human and mouse MDM2 coding regions revealed significant conservation at the nucleotide (80.3%) and amino acid (80.4%) levels. Although hMDM2 and mMDM2 bore little similarity to other genes recorded in current databases, the two proteins shared several motifs. These included a basic nuclear localization signal (Tanaka, 1990, FEBS Letters 271:41-46) at codons 181 to 185, several casein kinase II serine phosphorylation sites (Pinna, 1990, Biochem. et. Biophys. Acta. 1054:267-284) at codons 166 to 169, 192 to 195, 269 to 272, and 290 to 293, an acidic activation domain (Ptashne, 1988, Nature 355:683-689) at codons 223 to 274, and two metal binding sites (Harrison, 1991, Nature 353:715) at codons 305 to 322 and 461 to 478, neither of which is highly related to known DNA binding domains. The protein kinase A domain noted in mMDM2 (Fakharzadeh et al., 1991, EMBO J. 10:1565-1569) was not conserved in hMDM2.

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#### Example 2

To determine whether the hMDM2 protein could bind to human p53 protein in vitro, an hMDM2 expression vector was constructed from the cDNA clones. The hMDM2 expression vector was constructed in pBluescript SK+ (Stratagene) from overlapping cDNA clones. The construct contained the sequence shown in Figure 1 from nucleotide 312 to 2176. A 42 bp black bettle virus ribosome entry sequence (Dasmahapatra et al., 1987, Nucleic Acid Research 15:3933) was placed immediately upstream of this hMDM2 sequence in order to obtain a high level of expression. This construct, as well as p53 (El-Deriy et al., 1992, Nature Genetics, in press) and MCC (Kinzler et al., 1991, Science 251:1366-1370) constructs in pBluescript SK+, were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions.

Although the predicted size of the protein generated from the construct was only 55.2 kd (extending from the methionine at nucleotide 312 to nucleotide 1784), in vitro translated protein migrated at approximately 95 kilodaltons.

Ten  $\mu$ l of lysate containing the three proteins (hMDM2, p53 and MCC), alone or mixed in pairs, were incubated at 37°C for 15 minutes. One microgram (10  $\mu$ l) of p53 Ab1 (monoclonal antibody specific for the C-terminus of p53) or Ab2 (monoclonal antibody specific for the N-terminus of p53) (Oncogene Science), or 5  $\mu$ l of rabbit serum containing MDM2 Ab (polyclonal rabbit anti-hMDM2 antibodies) or preimmune rabbit serum (obtained from the rabbit which produced the hMDM2 Ab), were added as indicated. The polyclonal rabbit antibodies were raised against an *E. coli*-produced hMDM2-glutathione S-transferase fusion protein containing nucleotides 390 to 816 of the hMDM2 cDNA. Ninety  $\mu$ l of RIPA buffer (10 mM tris [pH 7.5], 1% sodium deoxycholate, 1% NP40, 150 mM NaCl, 0.1% SDS), SNNTE buffer, or Binding Buffer (El-Deriy et al., 1992, *Nature Genetics*, in press) were then added and the mixtures allowed to incubate at 4°C for 2 hours.

Two milligrams of protein A sepharose were added to each tube, and the tubes were rotated end-over-end at 4°C for 1 hour. After pelleting and washing, the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and the dried gels autoradiographed for 10 to 60 minutes in the presence of Enhance (New England Nuclear).

Figure 2 shows the co-precipitation of hMDM2 and p53. The three buffers produced similar results, although the co-precipitation was less efficient in SNNTE buffer containing 0.5 M NaCl (Figure 2, lanes 5 and 8) than in Binding Buffer containing 0.1 M NaCl (Figure 2 lanes 6 and 9).

In vitro translated hMDM2, p53 and MCC proteins were mixed as indicated above and incubated with p53 Ab1, p53 Ab2, hMDM2 Ab, or preimmune serum. Lanes 1, 4, 7, 10 and 14 contain aliquots of the protein mixtures used for immunoprecipitation. The bands running slightly faster than p53 are polypeptides produced from internal translation initiation sites.

The hMDM2 protein was not immunoprecipitated with monoclonal antibodies to either the C-terminal or N-terminal regions of p53 (Figure 2, lanes 2 and 3). However, when *in vitro* translated human p53 was mixed with the hMDM2 translation product, the anti-p53 antibodies precipitated hMDM2 protein along with p53, demonstrating an association *in vitro* (Figure 2, lanes 5 and 6). As a control, a protein of similar electrophoretic mobility from another gene (MCC (Kinzler et al., 1991, Science 251:1366-1370)) was mixed with p53. No co-precipitation of the MCC protein was observed (Figure 2, lanes 8 and 9). When an *in vitro* translated mutant form of p53 (175hm) was mixed with hMDM2 protein, a similar co-precipitation of hMDM2 and p53 proteins was also observed.

In the converse of the experiments described above, the anti-hMDM2 antibodies immunoprecipitated p53 when mixed with hMDM2 protein (Figure 2, lane 15) but failed to precipitate p53 alone (Figure 5, lane 13). Preimmune rabbit serum failed to precipitate either hMDM2 or p53 (Figure 2, lane 16).

#### Example 3

In order to ascertain the chromosomal localization of hMDM2, somatic cell hybrids were screened with an hMDM2 cDNA probe. A human-hamster hybrid containing only human chromosome 12 was found to hybridize to the probe. Screening of hybrids containing portions of chromosome 12 (Turc-Carel et al., 1986, Cancer Genet. Cytogenet. 23:291-299) with the same probe narrowed the localization to chromosome 12q12-14.

#### Example 4

Previous studies have shown that this region of chromosome 12 is often aberrant in human sarcomas. Mandahl et al., 1987, Genes Chromosomes & Cancer 1:9-14; Turc-Carel et al., 1986, Cancer Genet. Cytogenet. 23:291-299; Meltzer et al., 1991, Cell Growth & Differentiation 2:495-501. To evaluate the possibility that hMDM2 was genetically altered in such cancers, Southern blot analysis was performed.

Figure 3 shows examples of the amplification of the hMDM2 gene in sarcomas. Cellular DNA (5 μg) was digested with EcoRI, separated by agarose gel electrophoresis, and transferred to nylon as described by Reed and Mann (Nucl. Acids Res., 1985, 13:7207-7215). The cellular DNA was derived from five primary sarcomas (lanes 1-4, 6) and one sarcoma cell line (OsA-C1, lane 5). The filters were then hybridized with an hMDM2 cDNA fragment probe nucleotide 1-949 (see Figure 1), or to a control probe which identifies fragments of similar size (DCC gene, 1.65 cDNA fragment). Fearon, 1989, Science 247:49-56. Hybridization was performed as described by Vogelstein et al. (Cancer Research, 1987, 47:4806-4813). A striking amplification of hMDM2 sequences was observed in several of these tumors. (See Figure 3, lanes 2, 3 and 5). Of 47 sarcomas analyzed, 17 exhibited hMDM2 amplification ranging from 5 to 50 fold. These tumors included 7 to 13 liposarcomas, 7 of 22 malignant fibrous histiocytomas (MFH), 3 of 11 osteosarcomas, and 0 and 1 rhabdomyosarcomas. Five benign soft tissue tumors (lipomas) and twenty-seven carcinomas (colorectal or gastric) were also tested by Southern blot analysis and no amplification was observed.

#### Example 5

This example illustrates that gene amplification is associated with increased expression.

Figure 4A illustrates hMDM2 expression as demonstrated by Northern blot analysis. Because of RNA degradation in the primary sarcomas, only the cell lines could be productively analyzed by Northern blot. RNA was separated by electrophoresis in a MOPS-formaldehyde gel and electrophoretically transferred to nylon filters. Transfer and hybridization were performed as described by Kinzler et al. (*Nature 332*:371-374, 1988). The RNA was hybridized to the hMDM2 fragment described in Figure 3. Ten  $\mu$ g of total RNA derived, respectively, from two sarcoma cell lines (OsA-CL, lane 1 and RC13, lane 2) and the colorectal cancer cell line (CaCo-2) used to make the cDNA library (lane 3). Lane 4 contains 10  $\mu$ g of polyadenylated CaCo-2 RNA. RNA sizes are shown in kb. In the one available sarcoma cell line with hMDM2 amplification, a single transcript of approximately 5.5 kb was observed (Figure 4A, lane 1). The amount of this transcript was much higher than in a sarcoma cell line without amplification (Figure 4A, lane 2) or in a carcinoma cell line (Figure 4A, lane 3). When purified mRNA (rather than total RNA) from the carcinoma cell line was used for analysis, an hMDM2 transcript of 5.5 kb could also be observed (Figure 4A, lane 4).

Figure 4B illustrates hMDM2 expression as demonstrated by Western blot analysis of the sarcoma cell lines RC13 (lane 1), OsA-CL (lane 3), HOS (lane 4), and the carcinoma cell line CaCo-2 (lane 2).

Figure 4C illustrates hMDM2 expression as demonstrated by Western blot analysis of primary sarcomas. Lanes 1 to 3 contain protein from sarcomas with hMDM2 amplifications, and lanes 4 and 5 contain protein from sarcomas without hMDM2 amplification.

Western blots using affinity purified MDM2 Ab were performed with 50  $\mu$ g protein per lane as described by Kinzler et al. (Mol. Cell. Biol., 1990, 10:634-642), except that the membranes were blocked in 10% nonfat dried milk and 10% goat serum,

and secondary antibodies were coupled to horseradish peroxidase, permitting chemiluminescent detection (Amersham ECL). MDM2 Ab was affinity purified with a pATH-hMDM2 fusion protein using methods described in Kinzler et al. (*Mol. Cell. Biol. 10*:634-642, 1990). Non-specifically reactive proteins of about 75-85, 105-120 and 170-200 kd were observed in all lanes, irrespective of hMDM2 amplification status. hMDM2 proteins, of about 90-97 kd, were observed only in the hMDM2-amplified tumors. Protein marker sizes are shown in kd.

A protein of approximately 97 kilodaltons was expressed at high levels in the sarcoma cell line with hMDM2 amplification (Figure 4B, lane 3), whereas no expression was evident in two sarcoma cell lines without amplification or in the carcinoma cell line (Figure 4B, lanes 1, 2 and 4). Five primary sarcomas were also examined by Western blot analysis. Three primary sarcomas with amplification expressed the same size protein as that observed in the sarcoma cell line (Figure 4C, lanes 1-3), while no protein was observed in the two sarcomas without amplification (Figure 4C, lanes 4 and 5).

Expression of the hMDM2 RNA in the sarcoma with amplification was estimated to be at least 30 fold higher than that in the other lines examined. This was consistent with the results of Western blot analysis.

The above examples demonstrate that hMDM2 binds to p53 in vitro and is genetically altered (i.e., amplified) in a significant fraction of sarcomas, including MFH, liposarcomas, and osteosarcomas. These are the most common sarcomas of soft tissue and bone. Weiss and Enzinger, 1978, Cancer 41:2250-2266; Malawer et al., 1985, In: Cancer: Principles and Practice of Oncology, DeVita et al., Eds., pp. 1293-1342 (Lippincott, Philadelphia).

Human MDM2 amplification is useful for understanding the pathogenesis of these often lethal cancers.

MDM2 may functionally inactivate p53 in ways similar to those employed by virally encoded oncoproteins such as SV40 T-antigen, adenovirus E1B, and HPV E6. Lane and Bechimol, 1990. Genes and Development 4:1-8; Werness et al., 1990, Science

248:76. Consistent with this hypothesis, no sarcomas with hMDM2 amplification had any of the p53 gene mutations that occur commonly in other tumors. hMDM2 amplification provides a parallel between viral carcinogenesis and the naturally occurring genetic alterations underlying sporadic human cancer. The finding that expression of hMDM2 is correspondingly elevated in tumors with amplification of the gene are consistent with the finding that MDM2 binds to p53, and with the hypothesis that overexpression of MDM2 in sarcomas allows escape from p53 regulated growth control. This mechanism of tumorigenesis has striking parallels to that previously observed for virally induced tumors (Lane and Bechimol, 1990, Genes and Development 4:1-8; Werness et al., 1990, Science 248:76), in which viral oncogene products bind to and functionally inactivate p53.

#### Example 6

This example demonstrates that MDM2 expression inhibits p53-mediated transactivation.

To determine if MDM2 could influence the ability of p53 to activate transcription, expression vectors coding for the two proteins were stably transfected into yeast along with a p53-responsive reporter construct. The reporter consisted of a  $\beta$ -galactosidase gene under the transcriptional control of a minimal promoter and a multimerized human DNA sequence which strongly bound p53 in vitro (Kern, S.E., et al., Science 256:827-830 (1992). Reporter expression was completely dependent on p53 in this assay (Figure 5, compare bars a and c). MDM2 expression was found to inhibit p53-mediated transactivation of this reporter 16-fold relative to isogeneic yeast lacking MDM2 expression (Figure 5, compare bars a and b). Western blot analysis confirmed that p53 (53 kD) was expressed equivalently in strains with and without MDM2 (90 kD) (Figure 1, inset).

METHODS. The MDM2 expression plasmid, pPGK-MDM2, was constructed by inserting the full length MDM2 cDNA (Oliner, J.D., et al., Nature 358:80-83 (1992)) into pPGK (Poon, D. et al., Mol. and Cell.

Biol. 1111:4809-4821 (1991)), immediately downstream of the phosphoglycerate kinase constitutive promoter. Galactose-inducible p53 (pRS314SN, Nigro, J.M., et al., Mol. and Cell. Biol. 12:1357-1365 (1992)), lexA-VP16 (YVLexA, Dalton, S., et al., Cell 68:597-612 (1992)), and lexA (YLexA, YVLexA minus VP16) plasmids were used as indicated. The reporters were PG16-lacZ (Kern, S.E. et al., Science 256:827-830 (1992)) (p53-responsive) and pJK103 (Kamens, J., et al., Mol. Cell. Biol. 10:2840-2847 (1990)) (lexA-responsive). S. cerevisiae strain pEGY48 was transformed as described (Kinzler, K.W. et al., Nucl. Acids Res. 17:3645-3653 (1989)). Yeast strains represented by bars a-c were grown at 30°C to mid-log phase in selective liquid medium containing 2% raffinose as the carbon source, induced for 30 minutes by the addition of 2% galactose, harvested, and lysed as described (Kern, S.E. et al., Science 256:827-830 (1992)). The strains represented by bars d-f were treated similarly, except that the cells were induced in galactose for 4 hours to obtain measurable levels of  $\beta$ -galactosidase.  $\beta$ -galactosidase activities shown represent the mean of three to five experimental values (error bars indicate s.e.m.). Protein concentrations were determined by a Coomassie blue-based (bio-Rad) assay. The  $\beta$ -galactosidase assays were performed with AMPGD chemiluminescent substrate and Emerald enhancer (Tropix) according to the manufacturer's instructions. galactosidase activities of bars b and c are shown relative to that of bar A;  $\beta$ -galactosidase activities of bars e and f are shown relative to that of bar d. Western blots were performed as described (Oliner, J.D., et al., Nature 358:80-83 (1992), using p53 Ab1801 (lower panel, Oncogene Science) or MDM2 polyclonal antibodies (Oliner, J.D., et al., Nature 358:80-83 (1992)) (upper panel).

To ensure that this inhibition was not simply a general transcriptional down regulation mediated by the expression of the foreign MDM2 gene, a yeast strain was created that contained a different transcriptional activator (lexA-VP16, consisting of the lexA DNA binding domain fused to the VP16 acidic activation domain), a similar reporter (with a lexA-responsive site upstream of a  $\beta$ -galactosidase gene), and the same MDM2 expression vector. The results shown in Figure 1 (bars d & e) demonstrate that lexA-VP16 transactivation was unaffected by the presence of MDM2. Furthermore, MDM2 expression had no apparent effect on the growth rate of the cells.

#### Example 7

This example demonstrates the domains of p53 and MDM2 which interact with each other.

To gain insight into the mechanism of the MDM2-mediated p53 inhibition, the domains of MDM2 and p53 responsible for binding to one another were mapped. The yeast system used to detect protein-protein binding takes advantage of the modular nature of transcription factor domains (Keegan, L., et al., Science 231:699-704 (1986); Chien, C.-T., Proc. Natl. Acad. Sci. U.S.A. 88:9578-9582 (1991); Brent, R., et al., Cell 43:729-731 (1985); Ma, J., et al., Cell 55:4430446 (1988). Generically, if protein 1 (fused to a sequence-specific DNA binding domain) is capable of binding to protein 2 (fused to a transcriptional activation domain), then co-expression of both fusion proteins will result in transcriptional activation of a suitable reporter. In our experiments, the lexA DNA binding domain (amino acids 2-202) and the B42 acidic activation domain (AAD) were used in the fusion constructs. The reporter (Kamens, J., et al., Mol. Cell. Biol. 10:2840-2847 (1990); contained a lexA-responsive site upstream of a  $\beta$ galactosidase gene. As an initial control experiment, full length MDM2 was inserted into the lexA fusion vector, and full length p53, supplying its intrinsic activation domain was inserted into a non-fusion vector. The combination resulted in the activation of the lexAresponsive reporter, while the same expression constructs lacking either the MDM2 or p53 cDNA inserts failed to activate  $\beta$ -galactosidase (Table I, strains 1, 2, and 3). Thus, activation was dependent upon MDM2-p53 binding.

This assay was then applied to mapping the interaction domains of each protein. Full length cDNA fragments encoding MDM2 or p53 were randomly sheared by sonication, amplified by polymerase chain reaction, size fractionated, cloned into the appropriate fusion vectors and transfected into yeast along with the reporter and the full length version of the other protein.

METHODS. Full length MDM2 cDNA in pBluescript SK+(Stratagene) was digested with XhoI and BamHI to excise the entire insert. After agarose gel purification, the insert was sheared into random fragments by sonication, polished with the Klenow fragment of DNA polymerase I, ligated to catch linkers, and amplified by the polymerase chain reaction as described (Kinzler, K.W., et al., Nucl. Acids Res. 17:3645-3653 (1989)). The fragments were fractionated on an acrylamide gel into size ranges of 100-400 bp or 400-1000 pb, cloned into lexA(1-202)+PL (Ruden, D.M., et al., Nature 350:250-252 (1991)), and transfected into bacteria (XL-1 Blue, Stratagene). At least 10,000 bacterial colonies were scraped off agar plates, and the plasmid DNA was transfected into a strain of pEGY48 containing pRS314N (p53 expression vector) and pJK103 (lexA-responsive  $\beta$ -galactosidase reporter). Approximately 5,000 yeast clones were plated on selective medium containing 2% dextrose, and were replica-plated onto glalctose- and X-gal-containing selective medium. Blue colonies (17) appeared only on the plates containing the larger fragments of MDM2. The 17 isolated colonies were tested for blue color in this assay both in the presence and in the absence of galactose (p53 induction); all tested positive in the presence of galactose but only 2 of the 17 tested positive in its absence. MDM2-containing plasmid DNA extracted from the 17 yeast clones was selectively transferred to bacterial strain KC8 and sequenced from the lexA-MDM2 junction. The MDM2 sequences of the two p53independent clones are diagrammed in Fig. 6A. The MDM2 sequences of the remaining 15 p53-dependent clones coded for peptides ranging from 135 to 265 a.a. in length and began exclusively at the initiator methionine. Three of the MDM2 sequences obtained are shown at the top of Fig. 6B. The lower 6 sequences were genetically engineered (using the polymerase chain reaction and appropriate primers) into lexA(1-202)+PL and subsequently tested to further narrow the binding region.

Fragments of p53 were also cloned into pJG4-5, producing a fusion protein C-terminal to the B42 acidic activation domain and incorporating an epitope of hemagglutinin. The clones were transfected into a strain of pEGY48 already containing lex-MDM2 (plex-202+PL containing full length MDM2) and pJK103. The top three p53 sequences shown in Fig. 6C. were derived from yeast obtained by colony screening, whereas the lower three were genetically engineered to contain the indicated fragments.

The resultant yeast colonies were examined for  $\beta$ -galactosidase activity in situ. Of approximately 5000 clones containing MDM2 fragments fused to the lexA DNA

binding domain, 17 were found to score positively in this assay. The clones could be placed into two classes. The first class (two clones) expressed low levels of  $\beta$ galactosidase (about 5-fold less than the other fifteen clones) and  $\beta$ -galactosidase expression was independent of p53 expression (Figure 6A). These two clones encoded MDM2 amino acids 190-340 and 269-379, respectively. The region shared between these two clones overlapped the only acidic domain in MDM2 (amino acids 230-301). This domain consisted of 37.5% aspartic and glutamic acid residues but no basic amino acids. This acidic domain appears to activate transcription only when isolated from the rest of the MDM2 sequence, because the entire MDM2 protein fused to lexA had no measurable  $\beta$ -galactosidase activity in the same assay (Table I, strain 3). The other class (15 clones) each contained the amino terminal region of MDM2 (Figure 6B). The  $\beta$ -galactosidase activity of these clones was dependent on p53 co-expression. To narrow down the region of interaction, we generated six additional clones by genetic engineering. The smallest tested region of MDM2 which could functionally interact with full length p53 contained MDM2 codons 1 to 118 (Figure 6B). The relatively large size of the domain required for interaction was consistent with the fact that when small sonicated fragments of MDM2 were used in the screening assay (200 bp instead of 600 bp average size), no positively scoring clones were obtained.

In a converse set of experiments, yeast clones containing fragments of p53 fused to the B42 AAD were screened for lexA-responsive reporter expression in the presence of a lexA-MDM2 fusion protein. Sequencing of the 14 clones obtained in the screen revealed that they could be divided into three subsets, one containing amino acids 1-41, a second containing amino acids 13-57, and a third containing amino acids 1-50 (Figure 2C). The minimal overlap between these three fragments contained codons 13-41. Although this minimal domain was apparently necessary for interaction with MDM2, it was insufficient, as the fragments required 9-12 amino acids on either side of codons 13-41 for activity (Figure 6C). To further test the idea that the amino terminal region of p53 was required for MDM2 binding, we generated an additional yeast strain expressing

the lexA-DNA binding domain fused to p53 codons 74-393) and the B42 acidic activation domain fused to full length MDM2. These strains failed to activate the same lexA-responsive reporter (Table I, strain 8), as expected if the N-terminus of p53 were required for the interaction.

TABLE I

STRAIN NUMBER	p53 CONSTRUCT	MDM2 CONSTRUCT	ACTIVATION
1	p53*	Vector <sup>b</sup>	
2	p53*	lexA-MDM2b	+
3	Vector*	lexA-MDM2	-
4	p53*	lexA-MDM2 (1-118)	+
5	Vector	lexA-MDM2 (1-118) <sup>b</sup>	-
6	B42-p53 (1-41)°	lexA-MDM2*	+
7	Ь42-р53 (1-41)°	Vector	-
8	lexA-p53 (74-393) <sup>b</sup>	B42-MDM2°	_
9	p53 (1-137)*	lexA-MDM2*	-

The MDM2 and p53 proteins expressed in each strain, along with the relevant reporters, are indicated. Numbers in parentheses refer to the MDM2 or p53 amino acids encoded (absence of parentheses indicated full length protein, that is, MDM2 amino acids 1 to 491 or p53 amino acids 1 to 393). The lexa-responsive  $\beta$ -galactosidase reporter plasmid (pJK103, Kamens, J., et al., Mol. Cell. Biol. 10:2840-2847 (1990)) was present in all strains.

pRS314 vector (Nigro, J.M., et al., Mol. and Cell. Biol. 12:1357-1365 (1992).

plex(1-202)+PL vector, containing lexA DNA binding domain fused to insert (Ruden, D.M., et al., Nature 350:250-252 (1991).

pJG4-5 vector, containing B42 activation domain fused to insert.

 $<sup>^4(+)</sup>$  indicates that colonies turned blue following 24 hours of incubation on X-gal-containing selective medium, while (-) indicates that colonies remained white following 72 hours of incubation.

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Sequence analysis showed that all p53 and MDM2 fragments noted in Figure 6 were ligated in frame and in the correct orientation relative to the B42 and lexA domains, respectively. Additionally, all clones compared in Figure 6 expressed the relevant proteins at similar levels, as shown by Western blotting (Figure 7).

The most striking results of these mapping experiments was that the region of p53 required to bind MDM2 was almost identical to the previously identified acidic activation domain of p53 (amino acids 20-42) (Unger, T., et al., EMBO J. 11:1383-1390 (1992); Miller, C.W., et al., Proc. Am. Assoc. Cancer Res. 33:386 (1992). This suggested that MDM2 inhibits p53-mediated transcriptional activation by "concealing" the activation domain of p53 from the transcriptional machinery. If this were true, the p53 activation domain, in isolation from the rest of the p53 protein, should still be inhibitable by full length MDM2. To test this hypothesis, we produced a hybrid protein containing the p53 activation domain (codons 1-73) fused to the lexA-DNA binding domain. This construct exhibited strong transcriptional activation of a lexA-responsive reporter (Figure 8), as predicted from previous experiments in which the p53 activation domain was fused to another DNA binding domain (Fields, S., et al., Science 249:1046-1049 (1990); Raycroft, L., et al., Science 249:1049-1051 (1990)). The lexA-p53 DNA construct was stably expressed in yeast along with the full length MDM2 expression vector (or the vector alone). MDM2 expression resulted in a five-fold decrease in reporter activity, demonstrating that MDM2 can specifically inhibit the function of the p53 activation domain regardless of the adjacent protein sequences tethering p53 to DNA (Figure 8).

METHODS. Strains were grown to mid-log phase in 2% dextrose before induction of p53 expression for 2 hours by the addition of 2% galactose. The lex-p53 construct was identical to lex-VP16 (YVlexA, Dalton, S., et al., Cell 68:597-612 (1992)) except that VP16 sequences were replaced by p53 sequences encoding amino acids 1 to 73.

The results obtained in the experiments discussed herein raise an interesting paradox. If MDM2 binds to (Figure 6) and conceals (Figure 8) the p53 activation

domain from the transcriptional machinery, how could the lexA-MDM2-p53 complex activate transcription from the lexA-responsive reporter (Table I, strain 2)? Because the only functional activation domain in the lexA-MDM2-p53 complex of strain 2 is expected to be contributed by p53, one might predict that it would be concealed by binding to MDM2 and thereby fail to activate. A potential resolution of this paradox is afforded by knowledge that p53 exists as a homotetramer (Stenger, J.E., et al., Mol. Carcinogenesis 5:102-106 (1992); Sturzbecher, H.W. et al., Oncogene 7:1513-1523 (1992). Thus the activation noted in the lexA-MDM2-p53 complex could be due to the presence of four individual activation domains contributed by the p53 tetramer, not all of which were concealed by MDM2. As a direct test of this issue, the domain of p53 required for homo-oligomerization (Stenger, J.E., et al., Mol. Carcinogenesis 5:102-106 (1992); Sturzbecher, H.W. et al., Oncogene 7:1513-1523 (1992) (the C-terminus) was removed from the p53 expression construct, so that it consisted of only codons 1-137. This truncated p53 polypeptide retained the entire activation domain (as shown in Figure 8, bar a) and the entire domain required for interaction with MDM2 (Table I, strain 6). Yet, when allowed to interact with lexA-MDM2, no transactivation of the lexAresponsive reporter was observed (Table I, strain 9). Because p53 did not inhibit lexA-MDM2 binding to the lexA reporter (Table I, strain 2), the result of strain 9 is likely to be due to a direct inhibition of the isolated p53 activation domain by MDM2.

#### Example 8

This example illustrates the production and characterization of antibodies specific for MDM2 epitopes.

The antigen preparations used to intraperitoneally immunize female (BALB/c X C57BL/6)F1 mice comprised bacterially expressed, glutathione-column purified glutathione-S-transferase-MDM2 (GST-MDM2) fusion protein. (One preparation was further purified on a polyacrylamide gel and electroeluted.) The fusion protein contains a 16 kD amino terminal portion of human MDM2 protein (amino acids 27 to

168). For immunization, the fusion protein was mixed with Ribi adjuvant (Ribi Immunochem Research, Inc.).

Two mice were sacrificed and their spleen cells fused to SP2/0s myeloma cells (McKenzie, et al., Oncogene, 4:543-548, 1989). Resulting hybridomas were screened by ELISA on trpE-MDM2 fusion protein-coated microtiter wells. The trpE-MDM2 fusion protein contains the same portion of MDM2 as the GST-MDM2 fusion protein. Antigen was coated at a concentration of  $1 \mu g/ml$ .

A second fusion was performed as described except hybridomas were screened on electroeluted, glutathione purified GST-MDM2. Positive hybridomas from both fusions were expanded and single cell subcloned. Subclones were tested by Western Blot for specificity to the 55 kD trpE-MDM2 and the 43 kD GST-MDM2 fusion proteins.

Two Western Blot positive subclones (1F2 and JG3) were put into mice for ascites generation. The resulting ascites were protein A purified. Both purified monoclonal antibodies tested positive by Western Blot and immunoprecipitation for the 90 kD migrating MDM2 protein present in a human osteosarcoma cell line (OsA-CL), which overexpresses MDM2, and negative in the HOS osteosarcoma, which does not overexpress MDM2.

ED9 was protein G-purified from ascites and found to be specific in cryostat immunohistochemistry for MDM2 in osteosarcoma cells, as was IF2.

Example 9

This example demonstrates the expression and detection of MDM2 at the cellular level.

To evaluate MDM2 expression at the cellular level, we produced monoclonal antibodies against bacterially generated fusion proteins containing residues 27 to 168 of MDM2. (See example 8.) Of several antibodies tested, mAb IF-2 was the most useful, as it detected MDM2 in several assays. For initial testing, we compared proteins derived

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from OsA-CL, a sarcoma cell line with MDM2 amplification but without p53 mutation (Table II) and proteins from SW480, a colorectal cancer cell line with p53 mutation (Barak et al., EMBO 12:461-468 (1993)) but without MDM2 amplification (data not shown). Figure 9 shows that the mAb IF-2 detected an intense 90 kd band plus several other bands of lower molecular weight in OsA-CL extracts, and a much less intense 90 kd band in SW480 extracts. We could not distinguish whether the low molecular weight bands in OsA-CL were due to protein degradation or alternative processing of MDM2 transcripts. The more than 20-fold difference in intensity between the signals observed in OsA-CL and SW480 is consistent with the greater than 20-fold difference in MDM2 gene copy number in these two lines. Conversely, the 53 kd signal detected with p53-specific mAb 1801 was much stronger in SW480 than in OsA-CL consistent with the presence of a mutated p53 in SW480 (Fig. 9).

Cells grown on cover slips were then used to assess the cellular localization of the MDM2 protein. A strong signal, exclusively nuclear, was observed in OsA-CL cells with the IF-2 mAb and a weaker signal, again strictly nuclear, was observed in SW480 (Fig. 10). The nuclear localization of MDM2 is consistent with previous studies of mouse cells (Barak et al., EMBO 12:461-468 (1993)) and the fact that human MDM2 contains a nuclear localization signal at residues 179 to 186. Reactivity with the p53-specific antibody was also confined to the nuclei of these two cell lines (Fig. 10), with the relative intensities consistent with the Western blot results (Fig. 9).

The IF-2 mAb was then used (at 5  $\mu$ g/ml) to stain the seven primary sarcomas noted above. The nuclei of two of them (tumors #3 and #10) stained strongly (Fig. 11). Both of these tumors contained MDM2 gene amplification (Table II). In the five tumors without amplification, little or no MDM2 reactivity was observed (example in Fig. 11).

# TABLE II

	┝				
TUMOR	TUMOR	TYPE.	HDM2	P53	OVER-
-	, A		WILLIAM TOWN	ALTERATION	EXPRESSION
•	7 LE	11 L	ABSENT	DELETION/	NONE
^	<u> </u>			NEDANANGEMEN'I'	
j	CLE	I 4 E	ABSENT	CGC-CUC MUTATION;	p53
~	2			Arg(158)-His	•
	/-W	MFH	PRESENT	NONE OBSERVED	
4	M-8	MFH	Ancevim	and the second	MUM 2
u	:		ADDENT	DELETION	NONE
C	M-14	MFH	ABSENT	NONE OBSERVED	E 2
9	M-15	MFH	ABSENT		2.1.
7	M-16	NEW		UELLETION	N.T.
		ricii	ABSENT	NONE OBSERVED	MOME
8	M-17	MFII	ARGENII		NONE
c				NONE OBSERVED	N.T.
1	81-H	MFH	ABSENT	OVEREXPRESSED	4
10	M-20	MFH	PRESENT	NOW ORGANIE	rcd.
11	L-5	LIPOSABCOMA		MONE OBSERVED	MDM2
2	,	WHO	ABSENT	NONE OBSERVED	N.T.
71	١-١	LI POSARCOMA	ABSENT	AAC-AGC MUTATION;	N. T.
13	0-1	1 100010001		Asn(239)-Ser	
	Ć "	LI FUSARCOMA	PRESENT	NONE OBSERVED	E
					= .7

TABLE II (Cont.)

TUMOR	TUMOR	TYPE	MI)M2	P53	deno
•	QI		AMPLIFICATION	MITIATION	EXPRESSION
14	L-11	LIPOSARCOMA	ABSENT	NONE OBSERVED	
15	KL5B	LIPOSARCOMA	ARCENT		N. I.
			I NO COLUMN	CAG-UAG MUTATION; Gln(144)-Ston	r.T.
16	KL7	LI POSARCOMA	PRESENT	NONE OBSERVED	
17	KL10	LIPOSARCOMA	ABSENT	Canadago anon	. I. z
				WONE OBSERVED	N.T.
18	KE11	LIPOSARCOMA	ABSENT	GGT-GAT MUTATION; EXON 5 SPLICE DONOR SITE	N.T.
19	KL12	LIPOSARCOMA	ABSENT	NONE OBSERVED	
20	KI,28	I,I POSARCOMA	DDECENT		N.T.
			I WESTER	NONE UBSERVED	F.
21	KL30	LIPOSARCOMA	PRESENT	NONE OBSERVED	N.T.
22	S189	LIPOSARCOMA	PRESENT	NONE OBSERVED	8
23	S131B	LIPOSARCOMA	ABSENT	NONE OBGEDUES	N.I.
,	.00				N. T.
54	OSA-CL	MFH	PRESENT	NONE OBSERVED	MOM

• MFH= malignant fibrous histiocytoma

b as assessed by Southern blot

° as assessed by Southern blot, sequencing of exons 5-8, or immunohistochemical analysis

d as assessed by immunohistochemical analysis; N.T. = not tested

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#### SEQUENCE LISTING

#### (1) GENERAL INFORMATION:

- (i) APPLICANT: BURRELL, MARILEE
  HILL, DAVID E.
  KINZLER, KENNETH W.
  VOGELSTEIN, BERT
- (ii) TITLE OF INVENTION: AMPLIFICATION OF HUMAN MDM2 GENE IN HUMAN TUMORS
- (iii) NUMBER OF SEQUENCES: 5
- (iv) CORRESPONDENCE ADDRESS:
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  - (B) STREET: 1001 G STREET, N.W.
  - (C) CITY: WASHINGTON
  - (D) STATE: D.C.
  - (E) COUNTRY: USA
  - (F) ZIP: 20001
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

# (V1) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE: 07-APR-1993
- (C) CLASSIFICATION:

### (viii) ATTORNEY/AGENT INFORMATION:

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- (C) TELEX: 197430 BBMB UT

## (2) INFORMATION FOR SEQ ID NO:1:

- (1) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 64 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

- (v) FRAGMENT TYPE: N-terminal
- (V1) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
- (viii) POSITION IN GENOME:
  - (A) CHROMOSOME/SEGMENT: 17q
  - (x1) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln

1 10 15

Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu Asn Asn Val Leu 20 25 30

Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Leu Ser Pro Asp 35 40 45

Asp Ile Glu Gln Trp Phe Thr Glu Asp Pro Gly Pro Asp Glu Ala Pro 50 55 60

- (2) INFORMATION FOR SEQ ID NO:2:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 2372 base pairs
    - (B) TYPE: nucleic acid

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(C) STRANDEDNESS: double	
(D) TOPOLOGY: linear	
(ii) MOLECULE TYPE: cDNA	
(ill) HYPOTHETICAL: NO	
(iv) ANTI-SENSE: NO	
(vi) ORIGINAL SOURCE:	
(A) ORGANISM: Homo sapiens	
(H) CELL LINE: CaCo-2	
(viii) POSITION IN GENOME:	
(B) MAP POSITION: 12q12-14	
(ix) FEATURE:	
(A) NAME/KEY: CDS	
(B) LOCATION: 3121784	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:	
GCACCGCGCG AGCTTGGCTG CTTCTGGGGC CTGTGTGGCC CTGTGTGTCG GAAAGATGGA	60
GCAAGAAGCC GAGCCCJAGG GGCGGCCGCG ACCCCTCTGA CCGAGATCCT GCTGCTTTCG	120
CAGCCAGGAG CACCGTCCCT CCCCGGATTA GTGCGTACGA GCGCCCAGTG CCCTGGCCCG	180

GAG	AGTO	GAA	TGAT	rccc	CGA C	GCCC	AGGG	C GI	CGTC	CTT	C CGC	CAGTA	\GTC	AGT	ccc	GTG	240
AAG	GAAA	CTG	GGGZ	GTCT	TTG A	\GGGA	CCCC	C GA	CTCC	'AAGO	GCG	AAAA	CCC	CGGZ	TGG	TGA	300
GGA	GCAG	GCA		C TG													350
				1				5			F		10	sp G.	LY A	Ma	
		Thr		CAG			Ala					Thr					398
				CTT Leu		Lys					Val				Ly	8	446
				ATG Met	AAA	GAG				TAT	CTT				Il		494
				TTA Leu				Lys						TAT	TGT		542
			CTT	CTA										тст			590
ser	Asn	98 <b>A</b>	Leu	Leu	Gly	Asp	Leu 85	Phe	Gly	Val	Pro	Ser 90	Phe	Ser	Va]	1	

AAA	GAG	CAC	AGG	AAA	ATA	TAT	ACC	ATG	ATC	TAC	AGG	AAC	TTG	GTA	GTA	638
Lys	Glu	His	Arg	Lys	Ile	Tyr	Thr	Met	Ile	Tyr	Arg	Asn	Lei	ı Val	Val	
	95					100					105	i				
			CAG													686
Val	Asn	Gln	Gln	Glu	Ser	Ser	Asp	Ser	Gly	Thr	Ser	Val	Ser	Glu	Asn	
110					115					120					125	
															GAG	734
Arg	Cys	His	Leu	Glu	Gly	Gly	Ser	Asp	Gln	Lys	Asp	Leu	Val	Gln	Glu	
				130					135					140		
			GAG													782
Leu	Gln	Glu	Glu	Lys	Pro	Ser	Ser	Ser	His	Leu	Val	Ser	Arg	Pro	Ser	
			145					150					155			
			AGA			GCA	TTA	AGT	GAG	ACA	GAA	GAA	דממ	TCA	GAT	830
Thr	Ser	C														
		ser	Arg	Arg	Arg	Ala									Asp	
		160	Arg	Arg	Arg	Ala										
		160					Ile 165	Ser	Glu	Thr	Glu	Glu 170	Asn	Ser	Asp	
	TTA	160 TCT	GGT	gaa	CGA	CAA	Ile 165 AGA	Ser AAA	Glu CGC	Thr	Glu AAA	Glu 170 TCT	<b>Asn</b> GAT	Ser AGT	qaA TTA	878
	TTA Leu	160 TCT		gaa	CGA	CAA	Ile 165 AGA	Ser AAA	Glu CGC	Thr	Glu AAA	Glu 170 TCT	<b>Asn</b> GAT	Ser AGT	qaA TTA	878
	TTA	160 TCT	GGT	gaa	CGA	CAA	Ile 165 AGA	Ser AAA	Glu CGC	Thr	Glu AAA	Glu 170 TCT	<b>Asn</b> GAT	Ser AGT	qaA TTA	878
Glu	TTA Leu 175	TCT Ser	GGT Gly	GAA Glu	CGA Arg	CAA Gln 180	Ile 165 AGA Arg	Ser AAA Lys	Glu CGC Arg	Thr CAC His	Glu AAA Lys 185	Glu 170 TCT Ser	Asn GAT Asp	Ser AGT Ser	Asp ATT Ile	878
Glu	TTA Leu 175	TCT Ser	GGT Gly	GAA Glu GAT	CGA Arg GAA	CAA Gln 180 AGC	Ile 165 AGA Arg	Ser AAA Lys GCT	Glu CGC Arg	Thr CAC His	Glu AAA Lys 185 GTA	Glu 170 TCT Ser	Asn GAT Asp	Ser AGT Ser	ASP ATT Ile	878 926
Glu	TTA Leu 175	TCT Ser	GGT Gly	GAA Glu GAT	CGA Arg GAA	CAA Gln 180 AGC	Ile 165 AGA Arg	Ser AAA Lys GCT	Glu CGC Arg	Thr CAC His	Glu AAA Lys 185 GTA	Glu 170 TCT Ser	Asn GAT Asp	Ser AGT Ser	ASP ATT Ile	

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TGT	TGT	GAA	AGA	AGC	AGT	` AGC	AGT	GAA	TCT	' ACA	GGG	ACG	CC	TCC	AAT	974
															r Asn	
				210					215					22		
CCG	GAT	CTT	GAT	GCT	GGT	GTA	AGT	GAA	CAT	TCA	GGT	GAT	TGG	TTG	GAT	1022
Pro	Asp	Leu	Asp	Ala	Gly	Val	Ser	Glu	His	Ser	Gly	y Asp	Trį	Le	qaA u	
			225					230					235	5		
															TCT	1070
Gln	Asp	Ser	Val	Ser	Ąsp	Gln	Phe	Ser	Val	Glu	Phe	Glu	Val	. Glu	ı Ser	
		240					245					250	)			
															TCA	1118
Leu	qaA	Ser	Glu	Asp	Tyr	Ser	Leu	Ser	Glu	Glu	Gly	Gln	Glu	Leu	ı Ser	
	255					260					265	;				
															GAG	1166
Asp	Glu	Asp	qaA	Glu	Val	Tyr	Gln	Val	Thr	Val	Tyr	Gln	Ala	Gly	Glu	
270					275					280					285	
				TCA												1214
Ser	Asp	Thr	Asp	Ser	Phe	Glu	Glu	Asp	Pro	Glu	Ile	Ser	Leu	Ala	qaA	
				290					295					300		
				ACT '												1262
yr '	Lrp			Lhr	Ser	Сув	Asn	Glu	Met	Asn	Pro	Pro	Leu	Pro	Ser	
			305					310					315			

CAI	TGC	C AAC	AGA	TGT	TGG	GCC	CTT	CGT	GAG	AAT	TGG	CIT	CCI	' GAA	GAT	1310
Hıs	Cys	s Asr	ı Arç	Cys	Trp	Ala	Leu	Arg	g Glu	Asr.	Trp	Le	ı Pro	Glu	qaA ı	
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350					355					360					365	
ATA	GTG	AAT	GAT	TCC	AGA	GAG	TCA	TGT	GTT	GAG	GAA	AAT	GAT	GAT	AAA	1454
Ile	Val	Asn	Asp	Ser	Arg	Glu	Ser	Сув	Val	Glu	Glu	Asn	Asp	qaA	Lys	
				370					375					380		
ATT	ACA	CAA	GCT	TCA	CAA	TCA	CAA	GAA	AGT	GAA	GAC	TAT	TCT	CAG	CCA	1502
Ile	Thr	Gln	Ala	Ser	Gln	Ser	Gln	Glu	Ser	Glu	Asp	Tyr	Ser	Gln	Pro	
			385					390					395			
TCA	ACT	TCT	AGT	AGC	ATT	ATT	TAT	AGC	AGC	CAA	GAA	GAT	GTG	AAA	GAG	1550
Ser	Thr	Ser	Ser	Ser	Ile	Ile	Tyr	Ser	Ser	Gln	Glu	Asp	Val	Lys	Glu	
		400					405					410				
TTT	GAA	AGG	GAA	GAA	ACC	CAA	GAC .	AAA	gaa (	GAG .	AGT (	GTG	GAA	TCT .	AGT	1598
Phe	Glu	Arg	Glu	Glu	Thr	Gln	Asp	Lys	Glu	Glu	Ser	Val	Glu	Ser	Ser	
	415					420					425					

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TTG CCC CTT AF	AT GCC ATT GAA CCT	TGT GTG ATT TGT CAA G	GT CGA CCT 1646
Leu Pro Leu As	en Ala Ile Glu Pro	Cys Val Ile Cys Gln (	Sly Arg Pro
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		AAA ACA GGA CAT CTT A	
Lys Asn Gly Cy	's Ile Val His Gly	Lys Thr Gly His Leu M	let Ala Cys
	450	455	460
		AAA AGG AAT AAG CCC TO	
Phe Thr Cys Al	a Lys Lys Leu Lys	Lys Arg Asn Lys Pro C	ys Pro Val
46	5	470 4	75
		GTG CTA ACT TAT TTC CO	
	o Ile Gln Met Ile	Val Leu Thr Tyr Phe P	ro
480	485	490	
Thermenation on the			
TAGTIGACCT GTC	TATAAGA GAATTATAT	A TTTCTAACTA TATAACCCT	GGAATTTAGA 1844
CARCCIGARA ITI	ATTCACA TATATCAAA	TGAGAAAATG CCTCAATTC	CATAGATTTC 1904
المدارس المعاملة المارس المدارس المدار	A A COMPANIE A COMPANI		
TICICITIAG TAIL	AAFIGAC CTACTTTGG	AGTGGAATAG TGAATACTTA	CTATAATTTG 1964
ACTTGAATAT GTAG	CTCATC COMPAGE CO		
TOTAL GIA	SCICATE CIPTACACC	ACTCCTAATT TTAAATAATT	TCTACTCTGT 2024
CTTAAATGAG AAGT	المساحات الماليات	CTTAAATATG TATATGACAT	
	Action Intiffic	CITAAATATG TATATGACAT	TTAAATGTAA 2084
CTTATTATTT TTT	TGAGAC CGAGTCTTG	TCTGTTACCC AGGCTGGAGT	
		. ICIGITACCC AGGCTGGAGT	GCAGTGGGTG 2144
ATCTTGGCTC ACTG	CAAGCT CTGCCCTCCC	CGGGTTCGCA CCATTCTCCT	CCCTCACCCT 222
			CTL.L.ILMLSELT 22014

2264

2324

2372

CCCAATTAGC TTGGCCTACA GTCATCTGCC ACCACACCTG GCTAATTTTT TGTACTTT	TA
GTAGAGACAG GGTTTCACCG TGTTAGCCAG GATGGTCTCG ATCTCCTGAC CTCGTGAT	CC
GCCCACCTCG GCCTCCCAAA GTGCTGGGAT TACAGGCATG AGCCACCG	
(2) INFORMATION FOR SEQ ID NO:3:	
(i) SEQUENCE CHARACTERISTICS:	
(A) LENGTH: 491 amino acids	
(B) TYPE: amino acid	
(D) TOPOLOGY: linear	
(11) MOLECULE TYPE: protein	
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:	
Met Cys Asn Thr Asn Met Ser Val Pro Thr Asp Gly Ala Val Thr Thr	
1 5 10 15	
Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro	
20	
25 30	
Leu Leu Leu Lys Leu Lys Ser Val Gly Ala Gln Lys Asp Thr Tyr	
35	
40 45	
Thr Met Lys Glu Val Leu Phe Tyr Leu Gly Gln Tyr Ile Met Thr Lys	
50	
55 60	

Arg	Leu	Tyr	qaA	Glu	Lys	Gln	Gln	His	Ile	Val	Tyr	Сув	Ser	Asn	As
65					70					75					8
Leu	Leu	Gly	Asp	Leu	Phe	Gly	Val	Pro	Ser	Phe	Ser	Val	Lys	Glu	Hi
				85					90					95	
N		T.) -	<b></b>	<b></b>											
Arg	Lys	TIE	Tyr	Thr	Met	Ile	Tyr		Asn	Leu	Val	Val	Val	naA	Glr
			100					105					110		
Gln	Glu	Ser	Sar	Aen	Sar	G1++	Th.	C	**- *	_		_			
		115	Ser	пор	261	Gly	120	ser	vaı	Ser	Glu		Arg	Cys	Hie
							120					125			
Leu	Glu	Gly	Gly	Ser	Asp	Gln	Lys	gaA	Leu	Val	Gln	Glu	ī.au	Gla	G1.
	130				-	135	•				140	014	Deu	GIII	GIL
Glu	Lys	Pro	Ser	Ser	Ser	His	Leu	Val	Ser	Arg	Pro	Ser	Thr	Ser	Ser
145					150					155					160
Arg	Arg	Arg	Ala	Ile	Ser	Glu	Thr	Glu	Glu	Asn	Ser	Asp	Glu	Leu	Ser
				165					170					175	
Gly	Glu	Arg	Gln	Arg	Lys	Arg	His	Lys	Ser	Asp	Ser	Ile	Ser	Leu	Ser
			180					185					190		
Phe	Asp		Ser	Leu	Ala	Leu	Cys	Val	Ile	Arg	Glu	Ile	Сув	Сув	Glu
		195					200					205			
3	0 -	•	_	_											
		ser	Ser	Ser			Thr	Gly	Thr	Pro	Ser	Asn	Pro	Asp	Leu
	210					215					220				

370

Asp	Ala	Gly	/ Val	Ser	Glu	His	Ser	Gly	Asp	Trp	Leu	Asp	Gln	Asp	S <b>e</b>
225					230					235					24
Val	Ser	Asp	Gln	Phe	Ser	Val	Glu	Phe	Glu	Val	Glu	Ser	Leu	Asp	Se
				245					250					255	
Glu	qaA	Tyr	Ser	Leu	Ser	Glu	Glu	Gly	Gln	Glu	Leu	Ser	Asp	Glu	Ası
			260					265					270		
qaA	Glu	Val	Tyr	Gln	Val	Thr	VaI	Tyr	Gln	Ala	Gly	Glu	Ser	Asp	Thi
		275					280					285			
Asp	Ser	Phe	Glu	Glu	Asp	Pro	Glu	Ile	Ser	Leu	Ala	Asp	Tyr	Trp	Lys
	290					295					300				
Сув	Thr	Ser	Сув	Asn	Glu	Met	Asn	Pro	Pro	Leu	Pro	Ser	His	Cys	Asr
305					310					315					320
Arg	Cys	Trp	Ala	Leu	Arg	Glu	Asn	Trp	Leu	Pro	Glu	Asp	Lys	Gly	Lys
				325					330					335	
qaA	Lys	Gly	Glu	Ile	Ser	Glu	Lys	Ala	Lys	Leu	Glu	Asn	Ser	Thr	Gln
			340					345					350		
Ala	Glu		Gly	Phe	Asp	Val	Pro	Авр	Cys	Lys	Lys	Thr	Ile	Val	Asn
		355					360					365			
	_	_													
Asp	Ser	Arg	Glu	Ser	Сув	Val	Glu	Glu	Asn	Asp	Asp	Lys	Ile	Thr	Gln

375

380

WO 93/20238

Ala Ser Gln Ser Gln Glu Ser Glu Asp Tyr Ser Gln Pro Ser Thr Ser 385

Ser Ser Ile Ile Tyr Ser Ser Gln Glu Asp Val Lys Glu Phe Glu Arg

Glu Glu Thr Gln Asp Lys Glu Glu Ser Val Glu Ser Ser Leu Pro Leu
420 425 430

Asn Ala Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro Lys Asn Gly
435
440
445

Cys Ile Val His Gly Lys Thr Gly His Leu Met Ala Cys Phe Thr Cys
450
455
460

Ala Lys Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val Cys Arg Gln
465 470 475 480

Pro Ile Gln Met Ile Val Leu Thr Tyr Phe Pro

#### (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1710 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

VO 93/20238	PCT/US93/03199
Y () 73/ LULJO	[ (1/ (3/3/4317)

.....

- 46 -

(ii) MOLECULE TYPE: cDNA
(111) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(V1) ORIGINAL SOURCE:
(A) ORGANISM: Mus musculus
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 2021668
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
GAGGAGCCGC CGCCTTCTCG TCGCTCGAGC TCTGGACGAC CATGGTCGCT CAGGCCCCGT 60
CCGCGGGGCC TCCGCGCTCC CCGTGAAGGG TCGGAAGATG CGCGGGAAGT AGCAGCCGTC 120
TGCTGGGCGA GCGGGAGACC GACCGGACAC CCCTGGGGGA CCCTCTCGGA TCACCGCGCT 180
TCTCCTGCGG CCTCCAGGCC A ATG TGC AAT ACC AAC ATG TCT GTG TCT ACC 231
Met Cys Asn Thr Asn Met Ser Val Ser Thr
1 5 10
CAC CCM CCM CCA ACC ACC ACC
GAG GGT GCT GCA AGC ACC TCA CAG ATT CCA GCT TCG GAA CAA GAG ACT 279
Glu Gly Ala Ala Ser Thr Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr
20 25

CTG	GTT	r aga	CCZ	AA A	CCA	TTG	CTT	TTG	AAG	TTG	TTA	AAG	TCC	GTI	GGA	327
Leu	(Va)	Arg	y Pro	Lya	Pro	Leu	Leu	Lei	Lys	Le	ı Leı	ı Lyı	s Se	r Vai	l Gly	
			3 (					35					4 (			
GCG	CAA	AAC	GAC	ACT	TAC	ACT	ATG	AAA	GAG	ATT	ATA	TTT	TAT	ATT	GGC	375
Ala	Gln	Asn	. Asp	Thr	Tyr	Thr	Met	Lys	Glu	Ile	· Ile	Phe	туз	: Ile	Gly	
		45					50					55			•	
CAG	TAT	ATT	ATG	ACT	AAG	AGG	TTA	TAT	GAC	GAG	AAG	CAG	CAG	CAC	ATT	423
Gln	Tyr	Ile	Met	"hr	Lys	Arg	Leu	Tyr	Asp	Glu	Lys	Gln	Gln	His	Ile	
	60					65					70					
GTG	TAT	TGT	TCA	AAT	GAT	CTC	CTA	GGA	GAT	GTG	TTT	GGA	GTC	CCG	AGT	471
Val	Tyr	Cys	Ser	Asn	qaA	Leu	Leu	Gly	Asp	Val	Phe	Gly	Val	Pro	Ser	
75					80					85					90	
TTC	TCT	GTG	AAG	GAG	CAC	AGG	AAA	ATA	TAT	GCA	ATG	ATC	TAC	AGA	AAT	519
Phe	Ser	Val	Lys	Glu	His	Arg	Lys	Ile	Tyr	Ala	Met	Ile	Tyr	Arq	Asn	
				95					100				-	105		
TTA	GTG	GCT	GTA	AGT	CAG	CAA	GAC	TCT	GGC	ACA	TCG	CTG	AGT	GAG	AGC	567
Leu	Val	Ala	Val	Ser	Gln	Gln	Asp	Ser	Gly	Thr	Ser	Leu	Ser	Glu	Ser	
			110					115					120			
AGA	CGT	CAG	CCT	GAA	GGT	GGG .	AGT (	GAT	CTG .	AAG	GAT	CCT	TTG	CAA	GCG	615
						Gly										<b></b>
		125					130				-	125				

CC	A CC	'A GA	A GA	g aaj	A CC	r tca	TCI	TC	r gat	TT	ATT	rrci	' AGA	CTG	TCT	663
Pr	o Pr	0 G1	u Gl	u Lyi	s Pro	o Ser	Se	r Se	r Ası	P Le	u Il	e Sei	r Arg	Leu	Ser	
	14					145					15					
AC	C TC	A TC	T AG	A AGO	: AGN	, TCC	, y.com									
Th	r Se	r Se	r Arc	a Arc	7 220		. All	AGI	GAG	ACA	GAA	GAG	AAC	ACA	GAT	711
15:	5			, ,,,			116	s Sei	Glu	ı Thi	Glu	ı Glu	Asn	Thr	Asp	
	_				160	)				165	5				170	
GAG	G CT	A CC	r GGG	GAG	CGG	CAC	CGG	AAG	CGC	CGC	AGG	TCC	Cutv	<b>TCC</b>		
Glu	Le	ı Pro	o Gly	Glu	Arg	His	Arg	Lys	Ara	Ara	Arc	Ser	700		TTT	759
				175				•	180		, AL 9	Jer	Deu		Pne	
									100					185		
GAT	ccc	AGO	CTG	GGT	CTG	TGT	gag	CTG	AGG	GAG	ATG	TGC	AGC (	GGC (	GGC	807
Asp	Pro	Ser	Leu	Gly	Leu	Сув	Glu	Leu	Arg	Glu	Met	Сув	Ser	Glv	Glv	00,
			190					195				-	200	,	,	
ACG	AGC	AGC	AGT	AGC	AGC	AGC	AGC	AGC	GAG	TCC	ACA	GAG	ACG (	:cc :	rcg	855
Thr	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Glu	Ser	Thr	Glu	Thr	Pro	Ser	633
		205					210					215				
CAT	CAG	GAT	CTT	GAC	GAT	GGC	GTA	AGT	GAG	CAT	TCT	GGT (	GAT T	יפר ר	TY:	903
His	Gln	Asp	Leu	Asp	qaA	Gly	Val	Ser	Glu	His	Ser	Gly	Asp	Cva	T.A.	903
	220					225					230	7	р	<b>-</b> 75	<b>Deu</b>	
GAT	CAG	GAT	TCA	GTT '	TCT (	GAT (	CAG '	TTT.	AGC (	GTG (	GAA '	rr c	ם ממנ		n.c	051
qaA	Gln	qaA	Ser	Val	Ser	Asp (	Gln	Phe	Ser	Val	Glu	Phe	244 G	11 G	77	951
235					240					245		-116	G111 /			
														- 2	250	

TC	r cr	G GA	C TC	G GAZ	GAT	TAC	AGO	CTG	AGT	GAC	GA	A GG	G C	'AC	GAG	CTC	999
Se	r Le	u As	p Se	r Glu	ı Asr	Ту	r Se	r Leu	se:	r Ası	) G1	u Gl	y I	His	Gl	u Leu	
				255					26						265		
TC	A GA	r gag	G GAT	GAT	' GAG	GTC	TAT	. cgg	GTC	ACA	GTC	TA	r c	AG.	ACA	GGA	1047
Ser	Ası	Glı	Asp	gaA c	Glu	Val	Туг	Arg	Va]	Thr	Va.	1 ту	x G	ln	Thr	Gly	
			270					275						80			
GAA	AGC	GAT	ACA	GAC	TCT	TTT	GAA	GGA	GAT	CCT	GAG	ATT	r T	CC 7	ΓΤΑ	GCT	1095
Giu	Ser			qaA	Ser	Phe	Glu	Gly	Asp	Pro	Glu	ıIl	e S	er	Leu	Ala	
		285	•				290					29	5				
GAC	TAT	TGG	AAG	TGT	ACC	TCA	TGC	AAT	GAA	ATG	AAT	CCT	, cc	c c	TT	CCA	1143
Asp	Tyr	Trp	Lys	Сув	Thr	Ser	Сув	Asn	Glu	Met	Asn	Pro	o P	ro :	Leu	Pro	
	300					305					310						
TCA	CAC	TGC	AAA	AGA	TGC	TGG	ACC	CTT	CGT	GAG	AAC	TGG	CI	T C	CA	GAC	1191
Ser	His	Cys	Lys	Arg	Сув	Trp	Thr	Leu	Arg	Glu	Asn	Trp	Le	eu E	Pro	Asp	
315					320					325						330	
GAT	AAG	GGG	AAA	GAT	AAA	GTG	GAA	ATC '	TCT	GAA .	AAA	GCC	AA	A C	TG (	GAA	1239
Asp	Lys	Gly	Lys	Asp	Lys	Val	Glu	Ile	Ser	Glu	Lys	Ala	Ly	rs L	æu	Glu	
				335					340					3	45		
AAC	TCA	GCT	CAG	GCA (	GAA (	GAA (	GGC '	TTG (	GAT	GTG (	CT	GAT	GG	C AJ	4A <i>)</i>	<b>AA</b> G	1287
Asn	Ser	Ala	Gln	Ala	Glu	Glu	Gly	Leu .	Asp	Val	Pro	qaA	Gl	y L	ys	Lys	
			350					355					36			-	

CT	G AC	A GA	AG AA	T GA	TGC	T AA	A GAG	G CC	A TG	T GC	T GA	G GA	G GA	C AGO	GAG	1335
Le	u Th	r Gl	.u As	n As	p Al	a Lyı	s Gl	u Pr	о Су	s Al	a G1	lu G1	u As	p Se:	r Glu	
		36					37					37				
GA	G AA	G GC	C GA	A CAG	G ACC	ccc	CTG	TCC	CAC	G GAG	G AG	T GA	GAC	TAT	TCC	1383
Gl	ı Ly	s Al	a Gl	u Gli	n Thi	r Pro	Let	ı Se∶	r Gl	n Gl	u Se	r As	p Ası	р Туг	Ser	-505
	38	0				385					39			-		
CAJ	r cci	A TC	G ACT	TCC	AGC	AGC	ATT	GTT	TAT	AGC	AGO	CAZ	GAA	AGC	GTG	1431
Glr	Pro	Se:	r Thi	r Ser	Ser	Ser	Ile	Val	Ty	r Sei	Se:	r Gli	n Glu	Ser	Val	
395	i				400					405					410	
AAA	GAC	TTC	AAG	GAG	GAA	ACG	CAG	CAC	AAA	GAC	GAG	AGT	GTG	GAA	TCT	1479
Lys	Glu	Le.	Lys	Glu	Glu	Thr	Gln	His	Lys	Asp	Gli	ı Ser	. Val	Glu	Ser	
				415					420					425		
AGC	TTC	TCC	CTG	AAT	GCC	ATC	GAA	CCA	TGT	GTG	ATC	TGC	CAG	GGG	CGG	1527
Ser	Phe	Ser	Leu	Asn	Ala	Ile	Glu	Pro	Сув	Val	Ile	Сув	Gln	Gly	Arg	
			430					435					440			
CCT	AAA	AAT	GGC	TGC	ATT	GTT	CAC	GGC	AAG	ACT	GGA	CAC	CTC	ATG '	TCA	1575
Pro	Lys	naA	Gly	Сув	Ile	Val	His	Gly	Lys	Thr	Gly	His	Leu	Met	Ser	
		445					450					455				
TGT	TTC	ACG	TGT	GCA	AAG	AAG (	CTA.	AAA	AAA	AGA	AAC	AAG	CCC ·	TGC (	CA	1623
Сув	Phe	Thr	Сув	Ala	Lys	Lys	Leu	Lys	Lys	Arg	Asn	Lys	Pro	Сув	Pro	
	460					465					470					

GTG TGC AGA CAG CCA ATC CAA ATG ATT GTG CTA AGT TAC TTC AAC

Val Cys Arg Gln Pro Ile Gln Met Ile Val Leu Ser Tyr Phe Asn

480

485

TAGCTGACCT GCTCACAAAA ATAGAATTTT ATATTTCTAA CT

- (2) INFORMATION FOR SEQ ID NO:5:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 489 amino acids
    - (B) TYPE: amino acid
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: protein
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Cys Asn Thr Asn Met Ser Val Ser Thr Glu Gly Ala Ala Ser Thr

Ser Gln Ile Pro Ala Ser Glu Gln Glu Thr Leu Val Arg Pro Lys Pro
20 25 30

Leu Leu Leu Lys Leu Leu Lys Ser Val Gly Ala Gln Asn Asp Thr Tyr
35 40 45

Thr Met Lys Glu Ile Ile Phe Tyr Ile Gly Gln Tyr Ile Met Thr Lys
50 55 60

Arg		ту:	r Asp	Glu	1 Lys		Gln	His	Ile	• <b>Val</b>		Cys	Ser	Asn	<b>Asp</b>
Leu	Leu	Gl <sub>3</sub>	/ Asp	Val 85		Gly	Val	Pro	Ser 90		Ser	Val	Lys	Glu 95	His
Arg	Lys	Ile	100		Met	Ile	Tyr	Arg	Asn	Leu	Val	Ala	Val	Ser	Gln
Gln	Asp	Ser		Thr	Ser	Leu	Ser 120	Glu	Ser	Arg	Arg	Gln 125	Pro	Glu	Gly
Gly	Ser	Asp	Leu	Lys	Asp	Pro 135	Leu	Gln	Ala	Pro	Pro	Glu	Glu	Lys	Pro
Ser	Ser	Ser	Asp	Leu	Ile 150	Ser	Arg	Leu	Ser	Thr 155	Ser	Ser	Arg	Arg	Arg 160
Ser	Ile	Ser	Glu	Thr 165	Glu	Glu	Asn	Thr	<b>Asp</b>	Glu	Leu	Pro	Gly	Glu 175	Arg
His	Arg	Lys	Arg 180	Arg	Arg	Ser	Leu	Ser 185	Phe	Asp	Pro	Ser	Leu 190	Gly	Leu
Cys	Glu	Leu 195	Arg	Glu	Met		Ser 200	Gly	Gly	Thr	Ser	Ser 205	Ser	Ser	Ser
	Ser 210	Ser	Glu	Ser		Glu 215	Thr	Pro	Ser	His	Gln	Авр	Leu	Asp	qaA

215

220

22	5				230	)				235	3				1 Ser 240
Ası	⊃ G1:	n Ph	e Se	r Va. 24!		ı Phe	≘ Gli	ı Val	250		Leu	ı Asp	Sei	c Glu 255	ı Asp
Туг	Sei	r Le	u Se 26		o Glu	Gly	' His	265		Ser	Авр	Glu	270		Glu
Val	Tyr	275	y Vai	l Thr	Val	Tyr	Gln 280		Gly	Glu	Ser	Asp 285	Thr	qaA	Ser
Phe	Glu 290	Gly	, Yei	Pro	Glu	Ile 295	Ser	Leu	Ala	Asp	Tyr 300	Trp	Lys	Сув	Thr
Ser 305	Суз	Asn	Glu	Met	Asn 310	Pro	Pro	Leu	Pro	Ser	His	Сув	Lys	Arg	Сув 320
Trp	Thr	Leu	Arg	Glu 325	Asn	Trp	Leu	Pro	Asp 330	Asp	Lys	Gly	Lys	Asp 335	Lys
Val	Glu	Ile	Ser	Glu	Lys	Ala	Lys	Leu 345	Glu	Asn	Ser		Gln 350	Ala	Glu
Glu	Gly	Leu 355	Asp	Val	Pro .		Gly 360	Lys	Lys	Leu 1		Glu 365	Asn	Asp	Ala
Lys	Glu 370	Pro	Cys	∴la		31u .	Asp	Ser	Glu (	Glu 1	Lys	Ala (	Glu	Gln	Thr

375

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Pro	Leu	Ser	Gln	Glu	Ser	Asp	Asp	Tyr	Ser	Gln	Pro	Ser	Thr	Ser	Ser
385					390					395					400

Ser Ile Val Tyr Ser Ser Gln Glu Ser Val Lys Glu Leu Lys Glu Glu 405 410 415

Thr Gln His Lys Asp Glu Ser Val Glu Ser Ser Phe Ser Leu Asn Ala
420 425 430

Ile Glu Pro Cys Val Ile Cys Gln Gly Arg Pro Lys Asn Gly Cys Ile
435
440
445

Val His Gly Lys Thr Gly His Leu Met Ser Cys Phe Thr Cys Ala Lys
450 455 460

Lys Leu Lys Lys Arg Asn Lys Pro Cys Pro Val Cys Arg Gln Pro Ile 465 470 475 480

Gln Met Ile Val Leu Ser Tyr Phe Asn 485 -55International Application No: PCT/ /

MICROORG	GANISMS
Optional Sheet in connection with the microorganism referred is on	page
A. IDENTIFICATION OF DEPOSIT !	
Further deposits are identified on an additional shoot 1 1 2	
Name of depositary institution 4	
AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country)	12301 Parklawn Drive Rockville, Maryland 20852 United States of America
Date of deposit *	Accession Number *
March 11, 1993	HB 11290
B. ADDITIONAL INDICATIONS ! (leave blank if not applicable)	. This information is continued on a separate attached sheet
is sought a sample of the deposite available until the publication of	f the mention of the grant of the on which the application has been to be withdrawn, only the issue inated by the person requesting
D. SEPARATE FURNISHING OF INDICATIONS 4 (leave Man	A II not applicable)
The indications hated below will be submitted to the International — Accession Number of Copper (*)	Bureau later * (Specify the general nature of the Indications e.g.,
E. This sheet was received with the international application w	hen filed (to be checked by the receiving Office)
	(Authorized Officer)
The date of receipt (from the applicant) by the international	Bureau 19
ws6	(Authorized Officer)

Form PCT/RO/134 (January 1961)

	International Application No: PCT/ /
MICROOF	RGANISMS
Optional Sheet in connection with the microorganism referred to a	on page 10 Hne 19 of the description is
A. IDENTIFICATION OF DEPOSIT	m page , ine or the description !
Further deposits are identified on an additional sheet 🔀 a	
Name of depositary institution 4	
AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and countr 12301 Parklawn Drive	y) ·
Rockville, Maryland 20852, USA	
Date of deposit *	Accession Number 4
March 11, 1993	HB 11290
B. ADDITIONAL INDICATIONS 1 (leave blank if not applicable	e). This information is continued on a separate attached sheet
IEO Hybridama	
IF2 - Hybridoma	
C. DESIGNATED STATES FOR WHICH INDICATIONS AR	E MARE S (III has ladiowans as a second seco
	a made , (ii the molcations are not for an seeignated 2(6166)
D. SEPARATE FURNISHING OF INDICATIONS ! (Indived black	nk if not applicable)
The indications hated below will be submitted to the International "Accession Number of Deposit")	I Bureau later * (Specify the general nature of the Indications e.g.
E This sheet was received with the international application w	has filed the banks and the
- The state and received with the international application w	nen filed (to be checked by the receiving Office)
(	M (Nolmes
	(Authorized Officer)
The date of receipt from the sealers the s	
The date of receipt (from the applicant) by the international	Turosu 17
w96	
· <del>· · · ·</del>	(Authorized Officer)
	(mathematical Chicar)

4.	nternational Application No: PCT/ /
MICROOR	GANISMS
Optional Sheet in connection with the microorganism referred to on	page 10 Nne 19 of the description i
A. IDENTIFICATION OF DEPOSIT	
Further deposite are identified on an additional sheet 🗍 t	
Name of depositary inetitution *	
AMERICAN TYPE CULTURE COLLECTION	
Address of depository institution (including pools) code and country 12301 Parklawn Drive Rockville, Maryland 20852, USA	,•
Date of deposit * March 11, 1993	Accession Number 4 HB 11291
B. ADDITIONAL INDICATIONS ! (leave blank if not applicable	)). This information is continued on a separate attached shoot
ED9 - Hybridoma	
C. DESIGNATED STATES FOR WHICH INDICATIONS AR	E MADE * (It the indications are not for all designated States)
-	
D. SEPARATE FURNISHING OF INDICATIONS ! (leave ble	nk if not applicable)
The indications listed below will be submitted to the International "Accession Number of Deposit")	al Bureau leter 9 (Specify the general nature of the indications e.g.,
Accession number of Deposit /	
E. This sheet was received with the international application of	(Authorized Officer)
The date at receipt (from the applicant) by the International	ul Surecu +*
	(Authorized Officers

#### **CLAIMS**

- 1. A method of diagnosing a neoplastic tissue in a human comprising:

  detecting amplification of human MDM2 gene or elevated expression of a
  human MDM2 gene product in a tissue or body fluid isolated from a human, wherein
  amplification of the human MDM2 gene or elevated expression of human MDM2 gene
  product provides a diagnosis of neoplasia or the potential for neoplastic development.
  - 2. The method of claim 1 wherein gene amplification is detected.
- 3. The method of claim 1 wherein elevated expression of a gene product is detected, said gene product being mRNA.
- 4. The method of claim 1 wherein elevated expression of a gene product is detected, said gene product being human MDM2 protein.
- 5. The method of claim 3 wherein said mRNA is detected by Northern blot analysis by hybridizing mRNA from said tissue to a human MDM2 nucleotide probe.
- 6. The method of claim 5 wherein the human MDM2 nucleotide probe comprises nucleotides 1-2372 of human MDM2, as shown in Figure 1, or fragments thereof consisting of at least 14 contiguous nucleotides.
- 7. The method of claim 4 wherein human MDM2 protein is detected by Western Blot analysis by reacting human MDM2 proteins with antibodies which are immunospecific for MDM2 protein.
- 8. The method of claim 2 wherein the gene amplification is detected using polymerase chain reaction.
- 9. The method of claim 2 wherein amplification of the human MDM2 gene is detected by Southern blot analysis wherein the human MDM2 gene is hybridized with a nucleotide probe which is complementary to hMDM2 DNA.
- 10. The method of claim 2 wherein gene amplification is determined by comparing the copy number of hMDM2 in the tissue to the copy number of hMDM2 in a normal tissue of the human.

- 11. The method of claim 3 wherein elevated expression of mRNA is determined by comparing the amount of hMDM2 mRNA in the tissue to the amount of hMDM2 mRNA in a normal tissue of the human.
- 12. The method of claim 4 wherein elevated expression of hMDM2 protein is determined by comparing the amount of hMDM2 protein in the tissue to the amount of hMDM2 protein in a normal tissue of the human.
- 13. The method of claim 2 wherein gene amplification is detected when at least 3-fold more hMDM-2 DNA is observed in the tissue relative to a control sample comprising a normal tissue.
- 14. The method of claim 3 wherein elevated expression is detected when at least 3-fold more hMDM-2 mRNA is observed in the tissue relative to a control sample comprising a normal tissue.
- 15. The method of claim 4 wherein elevated expression is detected when at least 3-fold more hMDM2 protein is observed in the tissue relative to a control sample comprising a normal tissue.
  - 16. The method of claim 1 wherein the neoplasia is a sarcoma.
- 17. The method of claim 16 wherein the sarcoma is a liposarcoma, malignant fibrous histiocytoma, or osteosarcoma.
- 18. A cDNA molecule comprising nucleotides 1 to 2372, as shown in Figure 1, or fragments thereof, consisting of at least 14 contiguous nucleotides.
- 19. The cDNA molecule of claim 18 comprising the coding sequence of human MDM2.
  - 20. Human MDM2 protein substantially free of other human proteins.
- 21. A preparation of antibodies specifically immunoreactive with human MDM2 protein.
  - 22. The preparation of claim 21 wherein the antibodies are monoclonal antibodies.
- 23. A nucleotide probe comprising a sequence of at least 10 nucleotides which are complementary to nucleotides 1-2372 of human MDM2 gene, as shown in Figure 1.

- 24. A kit for detecting the amplification of a human MDM2 gene in a human tissue or body fluid sample comprising: a nucleic acid probe capable of hybridizing to said human MDM2 gene under conditions of high stringency, and instructions for determining said amplification.
- 25. A kit for detecting elevated expression of a human MDM2 mRNA in a human tissue or body fluid sample comprising: a nucleic acid probe capable of hybridizing to said mRNA, and written instructions for determining elevated expression of mRNA.
- 26. A kit for detecting elevated expression of a human MDM2 protein in a human tissue or body fluid sample comprising MDM2 protein-specific antibodies and written instructions for determining elevated expression of human MDM2 protein.
- 27. A method of treating a neoplastic cell or a cell having neoplastic potential, comprising:

administering to a cell a therapeutically effective amount of an inhibitory compound which interferes with the expression of human MDM2 gene.

- 28. The method of claim 27 wherein expression of the human MDM2 gene is inhibited by administering antisense oligonucleotides.
- 29. The method of claim 27 wherein expression of the human MDM2 gene is inhibited by administering triple-strand forming oligonucleotides which interact with DNA.
- 30. A method for identifying compounds which interfere with the binding of human MDM-2 to human p53, comprising:

binding a predetermined quantity of a first human protein which is detectably labelled to a second human protein;

adding a compound to be tested for its capacity to inhibit binding of said first and second proteins to each other;

determining the quantity of the first human protein which is displaced from or prevented from binding to the second human protein;

wherein the first human protein is MDM-2 and the second human protein is p53 or the first human protein is p53 and the second human protein is MDM-2.

- 31. The method of claim 30 wherein one of said two human proteins is fixed to a solid support.
- 32. The method of claim 30 wherein an antibody specifically immunoreactive with said second human protein is used to separate first human protein bound from unbound first human protein.
- 33. A method for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification, comprising:

administaring a polypeptide to tumor cells which contain a human MDM2 gene amplification, said polyptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide being capable of binding to human MDM2.

- 34. The method of claim 33 wherein said polypeptide comprises amino acids 1-41 of p53.
- 35. The method of claim 33 wherein said polypeptide comprises amino acids 13-57 of p53.
- 36. The method of claim 33 wherein said polypeptide comprises amino acids 1-50 of p53.
- 37. A method for inhibiting the growth of tumor cells which contain a human MDM2 gene amplification, comprising:

administering to tumor cells which contain a human MDM2 gene amplification a DNA molecule which expresses a polypeptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide being capable of binding to human MDM2.

38. The method of claim 37 wherein said polypeptide comprises amino acids 1-41 of p53.

- 39. The method of claim 37 wherein said polypeptide comprises amino acids 13-57 of p53.
- 40. The method of claim 37 wherein said polypeptide comprises amino acids 1-50 of p53.
- 41. A polypeptide consisting essentially of a portion of p53, said portion comprising amino acids 13-41 of p53, said polypeptide capable of binding to human MDM2.
  - 42. The polypeptide of claim 41 which comprises amino acids 1-41 of p53.
  - 43. The polypeptide of claim 41 which comprises amino acids 13-57 of p53.
  - 44. The polypeptide of claim 41 which comprises amino acids 1-50 of p53.
- 45. The preparation of claim 21 wherein the antibodies do not bind to other human proteins.
- 46. The preparation of claim 21 wherein the antibodies do not bind to human proteins of M<sub>r</sub> 75-85K, 105-120K, and 170-200K.
- 47. The preparation of claim 21 wherein the antibodies bind to the epitope bound by antibodies secreted by hybridoma IF2 (ATCC HB 11290).
- 48. The preparation of claim 21 wherein the antibodies bind to the epitope bound by antibodies secreted by hybridoma ED9 (ATCC HB 11291).
- 49. The method of claim 7 wherein the antibodies bind to the epitope on hMDM2 bound by antibodies secreted by hybridoma IF2 (ATCC HB 11290).
- 50. The method of claim 4 wherein human MDM2 protein is detected by immunohistochemistry.
- 51. The method of claim 50 wherein antibodies are employed in the immunohistochemistry which bind to an epitope on hMDM2 bound by the antibodies secreted by ED9 (ATCC HB 11291).
- 52. The method of claim 50 wherein antibodies are employed in the immunohistochemistry which bind to an epitope on hMDM2 bound by the antibodies secreted by IF2 (ATCC: HB 11290).

- 53. The method of claim 4 wherein human MDM2 protein is detected by immunoprecipitation.
- 54. A hybridoma cell having the identifying characteristics of ED9 (ATCC HB 11291).
- 55. A hybridoma cell having the identifying characteristics of IF2 (ATCC HB 11290).

## FIG. IA(I)

1	GC.	ACC	GCG	CGA	GCI	TGG	CTG	CTT	CTG	GGGC
									*	AG
84	GGC	CGC	GAC	CCC	TCT	GAC	CGA	GAT	CCT	GCTG
										GAAG
168	GTG	CCC	TGG	CCC	GGA	.GAG	TGG	AAT	GAT	cccc
	ACC									
252 1	GGA	GTC'	TTG	AĢG	GAC	CCC	CGA	CTC	CAA	.GCGC
	T	С	G			С	G			
336	CCT	ACT	GAT	GGT	GCT	GTA	ACC	ACC	TCA	CAGA
9	P	T	D	G	A	V	T	T	S	Q
	S		E			A	S			
	G			С		A	G		C	!
420		TTA	AAG				-		_	GACA
420 37		TTA L	AAG K	TCT	GTT	GGT	GCA	CAA	_	
	TTA' L			TCT	GTT	GGT	GCA A	CAA	AAA K N	GACA D
	TTA' L	L	K	TCT s	GTT V	GGT G	GCA A	<b>CAA</b> <b>Q</b> G	AAA K N	GACA D
37	TTA' L	L	K	TCT S C GAT	GTT V GAG	GGT G AAG	GCA A G	CAA Q G CAA	AAA K N	GACA D
<b>37 504</b>	TTA' L A G CGA'	L TTA	K TAT	TCT S C GAT	GTT V GAG	GGT G AAG	GCA A G	CAA Q G CAA	AAA K N C	GACA D ATTG
<b>37 504</b>	TTA' L A G CGA'	L TTA	K TAT	TCT S C GAT	GTT V GAG	GGT G AAG	GCA A G	CAA Q G CAA Q	AAA K N C	GACA D ATTG I
<b>37 504</b>	TTA' L A G CGA' R	L TTA L	K TAT Y	TCT S C GAT D	GTT V GAG E	GGT G AAG K	GCA A G CAA Q	CAA Q G CAA Q	AAA K N CAT H	GACA D ATTG I
37 504 65	TTA' L A G CGA' R	L TTA L	K TAT Y	TCT S C GAT D	GTT V GAG E	GGT G AAG K	GCA A G CAA Q	CAA Q G CAA Q	AAA K N CAT H	GACA D ATTG I
37 504 65 588	TTALL A G CGAL R	L TTA L G	K TAT Y GAG	TCT S C GAT D	GTT V GAG E	GGT G AAG K	GCA A G CAA Q	CAA Q G CAA Q	AAA K N CAT H G A	GACA D ATTG I ATGA
37 504 65 588	TTALL A G CGAL R	L TTA L G AAA	K TAT Y GAG	TCT S C GAT D	GTT V GAG E	GGT G AAG K	GCA A G CAA Q	CAA Q GAA Q TAT Y	AAA K N CAT H G A	GACA D ATTG I ATGA
37 504 65 588	TTA L A G CGA R GTG	TTA' L G AAA	K TAT Y GAG E	TCT S CAT D CAC H	GTT V GAG E AGG R	GGT G AAG K AAA K	GCA GCAA Q ATA I	CAA Q GAA Q TAT Y	AAA K N CAT H G A ACC T A	GACA D ATTG I ATGA
504 65 588 93	TTA L A G CGA R GTG	TTA' L G AAA	K TAT Y GAG E	TCT S CAT D CAC H	GTT V GAG E AGG AAC	GGT G AAG K AAA K	GCA A CAA Q ATA I C TGT	CAA Q CAA Q TAT Y	AAA K N CAT H G A ACC T A	GACA D ATTG I ATGA M

#### FIG. 1A(2)

CTGTGTGGCCCTGTGTGTCGGAAAGATGGAGCAAGA

AGCCGC GC TTCTC TCG TCGAGCT TG ACGAC CTTTCGCAGCCAGGAGCACCGTCCCTCCCCGGATTA

GTCGGAA ATGCGC G AAGTAG CC T CT GAGGCCCAGGGCGTCGTGCTTCCGCAGTAGTCAGTC

ACCGCG TTCTCCT C GCCTC C

GAAAACCCCGGATGGTGAGGAGCAGGCAAATGTGCA

M C

T
TTCCAGCTTCGGAACAAGAGACCCTGGTTAGACCAA
I P A S E Q E T L V R P

G
TATATTGTTCAAATGATCTTCTAGGAGATTTGTTTG
V Y C S N D L L G D L F
V

A T A G CT A G A---TCTACAGGAACTTGGTAGTAGTCAATCAGCAGGAAT
I Y R N L V V N Q Q E
A S -

TG T C T G C CA

GTGGGAGTGATCAAAAAGGACCTTGTACAAGAGCTTC
G G S D Q K D L V Q E L
L P L A P

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## FIG. 1A(3)

AGCCGAGCCCGAGGGG	BC 83	Human	nt
CATG CGCTCA G		Mouse	nt
GTGCGTACGAGCGCCC	2A 167	Human	nt .
GGGCGAGC GAGACC		Mouse	
CCCGTGAAGGAAACTG	iG 251	Human	nt
	G	Mouse	
ATACCAACATGTCTGT	'A 335	Human	nt
N T N M S V	7 8	Human	a.a.
		Mouse	a.a.
A		Mouse	nt
AGCCATTGCTTTTGAA	G 419	Human	nt
KPLLLK	36	Human	a.a.
		Mouse	a.a.
	G	Mouse	nt
AGTATATTATGACTAA		Human	nt
QYIMTK	64	Human	a.a.
		Mouse	a.a.
A C G T		Mouse	nt
GCGTGCCAAGCTTCTC			
G V P S F S	92		
		Mouse	a.a.
T C		Mouse	
CATCGGACTCAGGTAC	A 671	Human	nt
SSDSGT	120	Human	
		Mouse	a.a.
CA		Mouse	nt
AGGAAGAGAAACCTTC	CA 755	Human	nt
QEEKPS	148	Human	a.a.
P		Mouse	a.a.

## FIG. 18(1)

		T	G	A	A			TG	
756	TCT	TCA	CAT	TTG	GTT	rcT.	AGA	CCA	TCT
	S								
-								L	
	G	G	G	С	C G	G		G	GG
840	GGT								
177	G								
								R	R
	G	С	AGC	GGC	GGC	ACG	AGC.	A C	AGT
924	ATA'	TGT						TGT	GAA
205	I	C	-	-	-	_	-	C	E
	M		S	G	G	${f T}$	S	S	S
					${f T}$				
993	GTA	AGT	GAA	CAT	TCA	GGT	GAT	TGG	TTG
228	V	S	E	H	S	G	D	W	L
								С	
						_		_	
1077	TCA	GAA	GAT	TAT	AGC	CTT	AGT	GAA	GAA
		GAA	GAT	TAT	AGC	CTT	AGT	GAA E	
	TCA	GAA	GAT	TAT	AGC	CTT	AGT	GAA	GAA
	TCA S	GAA E	GAT D	TAT Y	AGC(	CTT L	AGT S	GAA E D	GAA
256	TCA S A	GAA E A	<b>GAT</b> D	TAT Y	AGC(	CTT L C	agt s	<b>GAA</b> <b>E</b> D	GAA E
256 1161	TCA S A GGG	GAA E A GAG	GAT D C AGT	TAT Y GAT	AGC( S ACA(	CTT L C GAT	AGT S TCA	GAA E D	GAA E
256	TCA S A GGG	GAA E A GAG	GAT D C AGT	TAT Y GAT	AGC(	CTT L C GAT	AGT S TCA	GAA E D	GAA E
256 1161	TCA S A GGG	GAA E A GAG	GAT D C AGT	TAT Y GAT	AGC( S ACA(	CTT L C GAT	AGT S TCA	GAA E D	GAA E
256 1161	TCA S A GGG	GAA E A GAG E	GAT D C AGT S	TAT Y GAT	AGC( S ACA(	CTT L C GAT	AGT S TCA S	GAA E D TTT F	GAA E GAA E
256 1161 284	TCA S A GGG G	GAA E A GAG E	GAT D C AGT S	TAT Y GAT D	AGC S ACA T	CTT L C GAT D	AGT S TCA S	GAA E D TTT F	GAA E A
256 1161 284 1245	TCA S A GGG G	GAA E A GAG E	GAT D C AGT S	TAT Y GAT D	AGC( S ACA( T	CTT L C GAT D	AGT S TCA S CAT	GAA E D TTT F	GAA E A AAC
256 1161 284	TCA S A GGG G	GAA E A GAG E	GAT D C AGT S	TAT Y GAT D	AGC S ACA T	CTT L C GAT D	AGT S TCA S CAT	GAA E D TTT F	GAA E A AAC N
256 1161 284 1245	TCA S A GGG G	GAA E A GAG E	GAT D C AGT S	TAT Y GAT D	AGC( S ACA( T	CTT L C GAT D	AGT S TCA S CAT	GAA E D TTT F	GAA E A AAC
256 1161 284 1245	TCA S A GGG G	GAA E A GAG E	GAT D C AGT S	TAT Y GAT D	AGC S ACA T CCA'	CTT L C GAT D	AGT S TCA S CAT	GAA E D TTT F	GAA E A AAC N
256 1161 284 1245	TCA S A GGG G	GAA E A GAG E T CCC P	GAT D C AGT S	TAT Y GAT D CTT L	AGC S ACA T CCA'	CTT L CGAT D	AGT S TCA S CAT H	GAA E D TTT F	GAA E GAA E AAC N K
256 1161 284 1245 312	TCA S A GGG G	GAA E AGAG E CCC P	GAT D C AGT S	TAT Y GAT D CTT L A GAG	AGC S ACA T CCA	CTT L CAT D	AGT S TCA S CAT H	GAA E D TTT F TGC CTG	GAA E AAC N K

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# FIG. 18(2)

												5
ACC!	rca:	CTZ	AGAZ	AGGZ	AGA	GCAZ	ATT	AGTO	GAGI	ACAC	SAAC	AA
		s						S				
-	D	J	10	1/	10	S	_			•	~	_
						٥						
					_							~
												G
TCT	GAT	AGT	ATTI	CCC	CTT	נככיו	CTT	GAT	IAA	AGC	CTGG	CT
S	D	S	I	S	L	S	F	D	E	S	L	Α
_	_	_	_						P			G
									~			
~		~		_	~	_		7.		$\sim$	_	,
		С										
AGA												
	S	S	S	S	E	S	T	G	${f T}$	P	S	N
S								E				H
					${f T}$				С	G		
GAT	יאכנ	ייי עב	רכים מ	ուսու							רממי	ւիւկո
D		D									•	
D	Z	ט	0	•	0	ט	δ	r	0	٧	בנ	£
_	_	_								_		
		G										
GGA	CAAC	JAA(	CTCI	CAC	TAE	SAAG	TAE	GATO	BAG	CATE	OTAT	CAA
G	Q	E	L	S	D	E	D	D	E	V	Y	Q
	H											R
G			G								G	${f T}$
GAA			_	<b>չ փփ</b> ո	יככיי	מיניים מיניים	Z C TT C	27. CT	חוף אניו	raaz	_	
E		P						D				
	ט	P	E	_	0	יד	A	ט	I	M	K	C
G												
	C	P	A				С			Α	С	
AGA	rgti	rgge	SCCC	CTTC	CGTC	SAGI	TAL	rgg	CTTC	CTC	SAAG	TA
R	С	W	A	${f L}$	R	E	N	W	L	p	E	D
			T								D	
			_								~	
	-	T	~	7\		λ		C		G		
3300											7.OM	1 3 IT
AAC												
N	S	T	Q	A	E	E	G		D	V	Ъ	ט
		7.						Τ.				

## FIG. 18 (3)

AAT' N	rca(	GAT		'AT'	TCT	839 176	Huma Huma	se nt in nt in a.a. se a.a.
CTG!	rgr C	GTA	I		GAG E	923 204	Huma Huma	
A CCGC P Q	GAT( D		GAT		C <b>GGT</b> <b>G</b>	992 227	Huma Huma	n nt n a.a. se a.a.
GAA(	GTT( V	G GAA! E	rct( s	G CTC L	GAC D	1076 255	Huma Huma	n nt n a.a. se a.a.
C GTT V	ACT			CAG	A GCA A T	1160 283	Huma Huma	
C ACT T	rca! s	rgc) C	AATO N	E E	ATG M	1244 311	Huma	e nt n nt n a.a. e a.a.
G <b>AAA</b> ( K	GGG G	AAA( K	GATI D	AAA K	T GGG G V	1228 339	Huma	
G C TGTI C G	AAA K	-	CTG ACTI T L		A GTG V E	1 <b>41</b> 2 367	Huma	in nt

## FIG. IC(1)

			G T	A		С		С		G
1413	AAT	GAT	TCC	AGA	GAG	TCA	TGT	GTT	GAG	GAA
368	N	D	S	R	E	S	C	V	E	E
			A	K		P		Α		
	_	A		G		_	С			G
1494	TCT	CAG	CCA	TCA	ACT	TCT.	AGT.	AGC	ATT	
395	S	Q	P	S	T	S	S	S	I	I
										V
	_						_	_	œ.	0
	C	3.0	<b>.</b>	ama.	~~~	m 0 m	C	C	_	G
1578	GA?	\G.		GTG					_	
423	E		S	V	E	S	S	L F	P S	L
	D							Г	5	
	${f T}$	\		G	т		С	С		TA
1662	GTC	CAT	ی د تا	AAA	ACA	GGA	CAT	CTT.	ATG	GCC
451	v	H	G	K	T	G	Н	L	M	A
										S
		G		_ C						G
1746	AGA									
479	R	Q	P	I	Q	M	I	V	L	T
										S
1830	TAA	CCC	TAG	GAA'	TTT.	AGA	CAA	CCT	GAA	ATT
1914	TTA									
1998	ACT									
2082	ATG!	<b>TAA</b>	CTT	ATT	ATT	$\mathbf{T}\mathbf{T}\mathbf{T}$	TTT	GAG	ACC	GAG
2166	CTC!	rgc	CCT	CCC	CGG	GTT	CGC	ACC	ATT	CTC
2250	TAA'	TTT	TTT	GTA	CTT	TTA	GTA	GAG	ACA	GGG
2334	CTC	GGC	CTC	CCA	AAG	TGC	TGG	GAT	TAC	AGG

### FIG. IC(2)

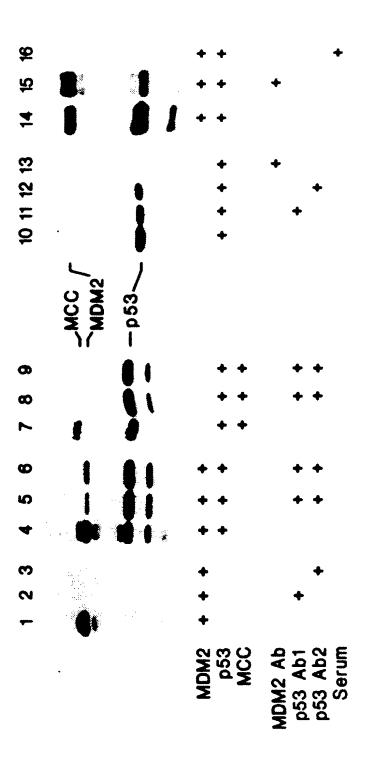
AA N	CAGC	GAT D	GAT D	AAA K	ATT.	ACA T	CAA( Q	GCT' A	rca s	CAA: Q	rcac
					AGC				G-		Α
TA	TAGO	AGC	CAA	GAA	GAT	GTG	AAA	GAG'	TTT	GAA	AGGG
Y	S	S	Q	E	D	V	K	E	F	$\mathbf{E}$	R
			~		S				L	-	K
		С		A			С	С	G	G	G
AA	TGCC										
	TGCC A	ATT	GAA	CCT	TGT	GTG	ATT'	TGT	CAA	GGT	CGAC
N	<b>A</b> T C	ATT I	GAA E	CCT P	TGT C	GTG V	ATT'	rgt C A	Q Q	GGT( G A	CGAC R
n	T C	ATT I : G	GAA E .TGT	CCT P GCA	TGT C AAG	GTG V AAG	ATT I CTA	TGT C A AAG	CAA Q AAA	GGT( G A AGG	CGAC R C AATA
n	<b>A</b> T C	ATT I : G	GAA E .TGT	CCT P GCA	TGT C AAG	GTG V AAG	ATT I CTA	TGT C A AAG	CAA Q AAA	GGT( G A AGG	CGAC R C AATA

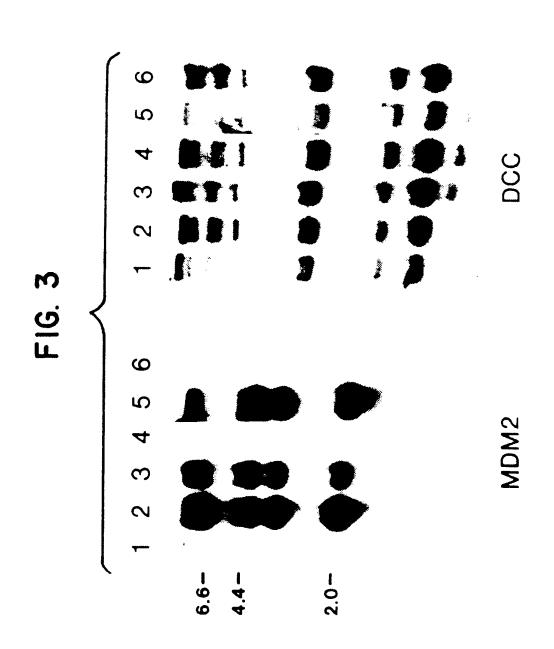
C AA C CTCA A A T
TATTTCCCCTAGTTGACCTG---TCTATAAGAGAATT
Y F P
N

# FIG. 1C(3)

G G C AAGAAAGTGAAGACTAT Q E S E D Y D	1493 Hur 394 Hur	ise nt nan nt nan a.a. ise a.a.
G GC  AAGAAACCCAAGACAAA E E T Q D K H	1577 Hur 422 Hur	nse nt nan nt nan a.a. use a.a.
C CTAAAAATGGTTGCATT P K N G C I	1661 Hu 450 Hu	use nt man nt man a.a. use a.a.
G C AGCCCTGCCCAGTATGT K P C P V C	1745 Hu 478 Hu	use nt man nt man a.a. use a.a.
T * ATATATTTCTAACTATA	1829 Hu 491 Hu	use nt man nt man a.a. use a.a.
ACATAGATTTCTTCTCT GCTCATCCTTTACACCA ATGTATATGACATTTAA TCTTGGCTCACTGCAAG CTGCCACCACACCTGGC CCTCGTGATCCGCCAC	1997 Hu 2081 Hu 2165 Hu 2249 Hu 2333 Hu	man nt

FIG. 2





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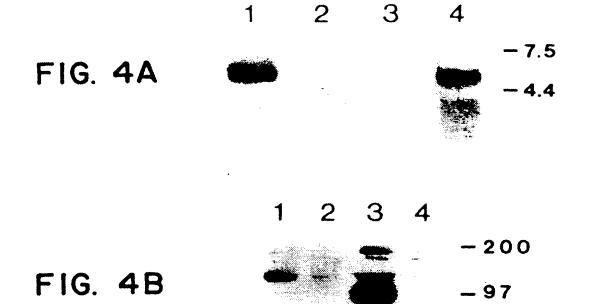
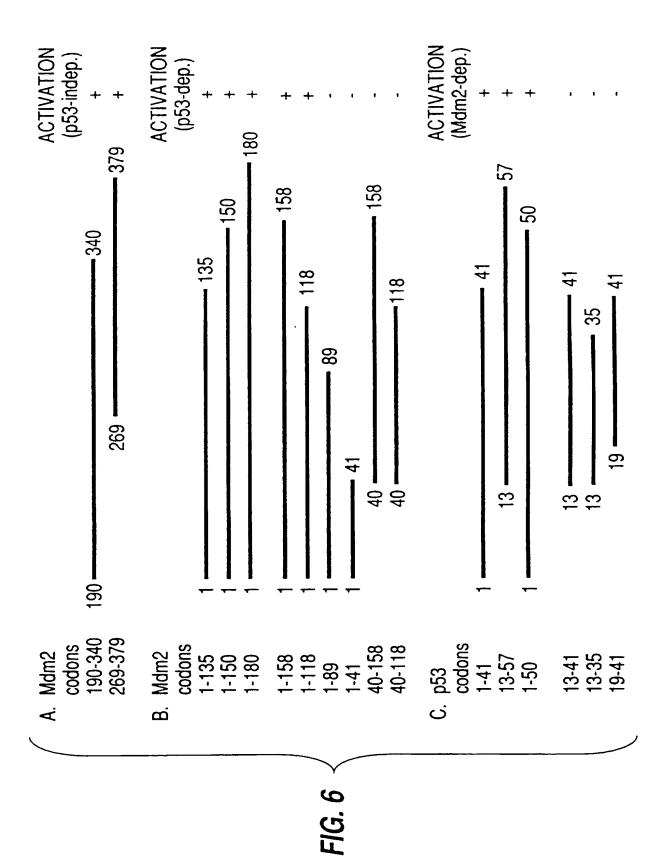


FIG. 4C - 200 - 97

06 lex-vp16 lex-vp16 Mdm2: F16. 5 p53 Transactivator: Mdm2: 150 50 100 Relative Beta-Galactosidase Activity

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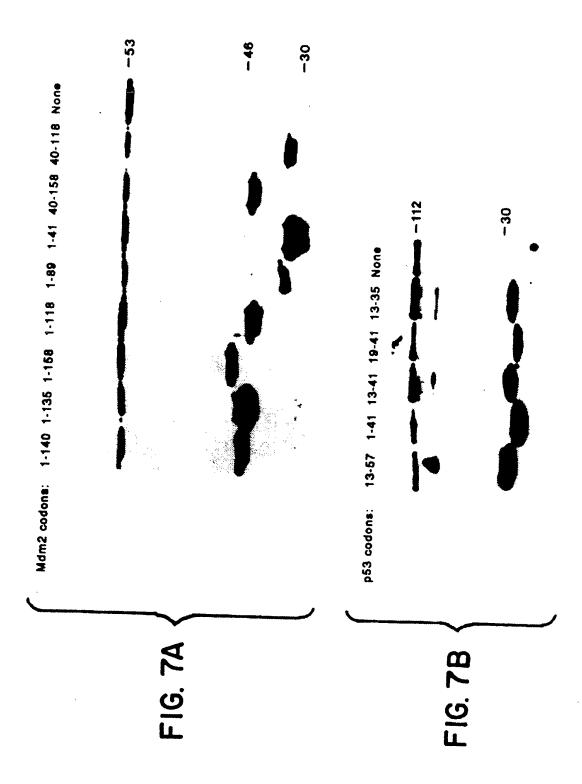
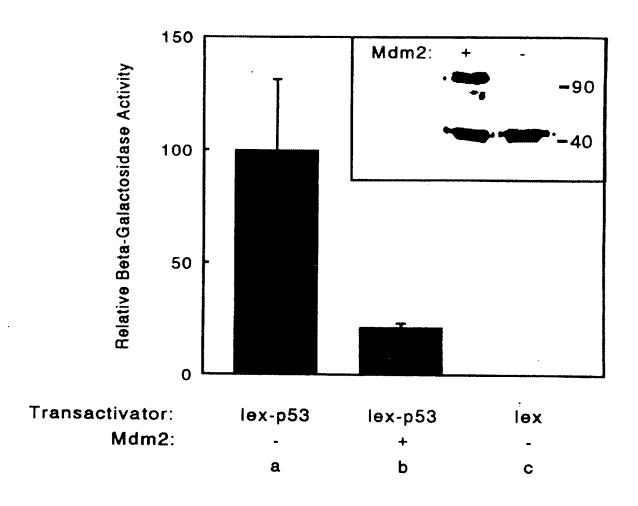
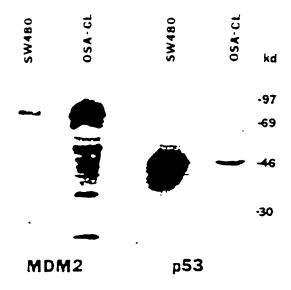


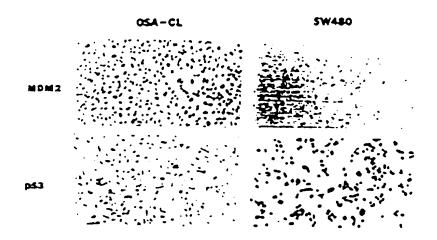
FIG. 8



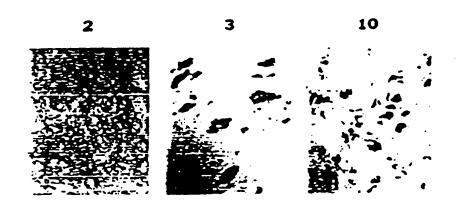
### FIGURE 9



#### FIGURE 10



## FIGURE 11



#### **SUBS**TITUTE SHEET

LOASS	FICATION OF SUBJ	ECT MATTER (If several classification	a symbols apply, indicate all) <sup>4</sup>	
		t Classification (IPC) or to both National		
Int.Cl	1. 5 C12Q1/68	G01N33/574;	C07H21/O0	
IL FIELD	S SEARCHED			
		Minimum Decu	mentation Searched	
Classifics	dica System		Classification Symbols	
Int.Cl	. 5	C12Q		
		Documentation Searched oth	er than Minimum Decumentation	
		to the Extent that ruch Document	ts are included in the Fields Searched <sup>4</sup>	
				i
III. DOCU	MOENTS CONSIDERE	D TO BE RELEVANT		
Casegory *	Citation of Do	ocument, II with indication, where approp	prints, of the relevant passages <sup>(2</sup>	Relevant to Claim No.13
A	EMBO JOL		4 OVECDE OF	1-55
		, no. 6, 1991, EYNSḤAN 565 - 1569	N, UXFURD GB	
		ADEH ET AL. 'Tumorigin	nic potential	
		ted with enhanced expr		
		at is amplified in a m	mouse tumor	
	cell lin			
		n the application whole document		
	see the	Auole document		
٨	J BIOL O	CHEM 263 (32). 1988. 1	17150-17158.	-
	SNYDER L	L C ET AL. 'À GENE AMP	PLIFIED IN A	
		RMED MOUSE CELL LINE U		
		TRANSCRIPTIONAL PROCE A NUCLEAR PROTEIN.'	122 TUR YUN	
	LINCODES	A HOULLAN PROJECT.		
	İ		-/	
* Seede	L categories of ched dec	. 10	"I" later document published after the interes	tional filing date
"A" de	Cament defining the gen	eral state of the art which is not	or priority date and not in conflict with the cited to understand the pranciple or theory	le application but
001	estidented to be of partico	ins relevance shed on or after the international	invention .	
GLI C	ing date		"X" document of particular relevances the cital cannot be considered nevel or cannot be o	met (avention ) messioned to
W	och is cited to establish t	r doubts on priority claim(s) or the publication date of another	invalve an inventive step.  'Y' document of particular relevance; the clair	med invention
	tition of other special rel Comment referring to an o	nson (as specified) eral disclasure, me, cobiletten er	cappet be established to involve an invest document to combined with one or more of	Ive step when the
•0	M Date .		ments, such combination being obvious to in the art.	
in	er then the printity date	to the international filing date but ordained	"A" document member of the same paint fan	وللا
IV. CERT	FICATION			
Date of the	Actual Completion of the	he Isternational Search	Date of Mailing of this Interesponsi Sear	th Report
	17 SEPTEMB	FR 1993	1	<b>3 1.</b> 10. 93
				, ii 10. 33
Internetor	i Searching Authority		Signature of Allthorized Officer	
	EUROPEA	N PATENT OFFICE	MOLINA GALAN E.	

III. DOCUMI	ENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)	
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
<b>A</b>	CELL GROWTH & DIFFERENTIATION vol. 1, 1990, pages 571 - 580 HINDS ET AL. 'Mutant p53 DNA clones from human colon carcinomas cooperate with ras in transforming primary rat cells: a comparison of the "hot spot" mutant phenotypes' cited in the application	
<b>A</b>	EP,A,O 341 904 (TEMPLE UNIVERSITY) 15 November 1989 see abstract	30-33
A	US,A,4 968 603 (SLAMON ET AL.) 6 November 1990	-
P,X	NATURE vol. 358, 2 July 1992, LONDON GB pages 80 - 83 OLINER ET AL. 'Amplification of a gene encoding a p53 associated protein in human sarcomas' see the whole document	1,18,20
P,X	CELL 69 (7). 1992. 1237~1245.  MOMAND J ET AL. 'THE MDM -2 ONCOGENE PRODUCT FORMS A COMPLEX WITH THE'	20

#### ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9303199 SA 73548

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0341904	15-11-89	JP-A- 2013400		
US-A-4968603	06-11-90	None		
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